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UNIVERSITY OF ALBERTA

BIOCHEMICAL EVENTS DURING AND FOLLOWING LOBLOLLY PINE SEED GERMINATION

BY

MARALEE GROOME

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

IN

PLANT MOLECULAR BIOLOGY

DEPARTMENT OF BOTANY

Edmonton, Alberta

FALL, 1991

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TO MY FAMILY

ABSTRACT

The correlation between lipid and protein reserve hydrolysis of loblolly pine (*Pinus taeda* L.) seed and changes in protein synthesis and enzyme activities was studied. Seeds were incubated at 30°C for up to 12 days following stratification, then megagametophytes and embryos were assayed for lipid and protein content after each day of imbibition. The megagametophyte of loblolly pine seed functions as a nutritional source for the embryonic axis during early seedling growth after the consumption of embryonic reserves. Comprised of 20% lipid and 12% buffer-insoluble storage protein, the megagametophyte quickly hydrolyzed these reserves upon imbibition to provide the embryonic axis with sugars and amino acids. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and scanning densitometry indicated that crystalloid-like proteins comprised 85% of the insoluble protein storage reserves. The soluble fractions from both tissues were labelled with [^{35}S]-methionine, and incorporation was studied by one and two-dimensional electrophoresis. Proteins were found to belong to different subsets defined by temporal patterns of synthesis. Changing catalase and isocitrate lyase activities were determined for the study period. Both enzymes increased in activity following seed germination. Peaks in the activities of both enzymes corresponded well to maximum lipid hydrolysis in the megagametophyte. The developmental shifts in protein synthesis and enzyme

activities accompanying seed reserve hydrolysis suggest that the biochemical events are tightly controlled.

Embryos were excised from the megagametophyte of mature seed to determine whether the embryo plays a role in this regulation. Megagametophyte halves were incubated independent of the embryo at 30°C on agar, then assayed for developmental changes in lipid and protein reserves, as well as for activities of catalase and isocitrate lyase. Removal of the embryo did not affect the rate of hydrolysis of proteins or lipids, but appeared to be required to achieve optimal catalase and isocitrate lyase activities. Removal of the embryo greatly affected the pattern of protein synthesis.

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I. INTRODUCTION

1.1 THE STUDY OF GENE EXPRESSION

The publication of Watson and Crick's paper (1953) on the structure of genetic material began the exciting era of molecular biology. Since then the study of information storage, processing, and gene expression have become significant areas of research. An important function of the genome in an organism is to direct protein synthesis. During development, the capacity to synthesize proteins becomes restricted in a precisely controlled way, each cell only utilizing a minute part of its genetic information. This differential gene expression relies on mechanisms which select portions of the genome to direct protein synthesis. It is no wonder that for the past four decades, the study of gene expression and the mechanisms restricting expression has held the interest of many people. While research involving human and animal gene expression and regulation has accumulated rapidly, parallel studies in plant systems are behind. Within the plant kingdom itself, information generated on gene expression is noticeably limited in gymnosperms.

1.2 SIGNIFICANCE OF LOBLOLLY PINE

As the world population continues to increase, so does the demand for wood, wood pulp and their products. Developed countries consume vast quantities of wood products such as paper and fiberboard while many less developed

countries still rely on wood for energy and construction. In 1986, wood-yielding forests constituted an estimated 90% of the total terrestrial biomass (Simpson & Ogarsaly 1986). Each year this figure shrinks drastically to meet the world's needs (Myers 1984). Trees are a renewable resource, but only if wisdom is used to ensure propagation; care must be taken to guarantee the replenishment of deforested areas in the quickest way possible.

Until 1950, all wood harvested came from natural forests. Now, many methods of forestation have been implemented such as reforestation, afforestation, and tree-planting which help to supplement the wood supply. Forest industries now rely heavily on plantations as alternative sources of raw materials. Because of the high demand for these raw materials, there is a continual search for potential plantation species. Many species of pine are grown in plantations because they grow very quickly under cultivation.

Loblolly pine (*Pinus taeda* L.) or "oldfield pine" occurs naturally in the coastal plains of the United States from Delaware to Central Florida, Eastern Texas, Southern Arkansas, and Southern Tennessee (U.S. Department of Agriculture 1974). As a plantation species in these areas, loblolly pine has become very common. In 1982, 60% of the plantings in the southern states were loblolly pine (Zobel 1982). The species is used extensively for timber production, newsprint, writing paper and food containers.

Once used for only a few restricted purposes, economic emphasis has shifted to loblolly in contributing nearly all kinds of fiber and solid-wood products.

As substitute forests proliferate, so does the demand for a dependable supply of good quality seed. Most of this seed is obtained from cultivated seed orchards which insure high quality and a dependable supply. Seed orchards are established by planting either clones (grafts) or seedling progeny from selected trees (Sheperd 1986). They are categorized as first- or second-generation orchards depending on the level of genetic improvement attained. The trees designated to take grafts or to establish seedlings from, are either the best selected from a normal population (first-generation select) or the best chosen from first-generation select trees (second-generation select). A stand of trees within an orchard is usually somewhat isolated from others to reduce the variability in pollen rain, thus ensuring that the genetic quality of seed is maintained.

Even if large quantities of good seed can be obtained to supply a plantation's need, seed must be stored in a way that maintains viability. Subsequent germination even of properly stored seed often presents problems to plantation managers. The highest mortality usually occurs shortly after germination (U.S. Department of Agriculture 1958) and this increases costs and reduces seedling availability for planting. The ability of seed to germinate and to establish seedlings is thus very important. These problems generate a

need for more basic biochemical and molecular information at the level of the seed during germination and early seedling growth.

One advantage of using loblolly pine as a system to study the processes occurring during germination and early seedling growth is the availability of large quantities of seed from first-generation select trees. As the megagaemtophyte is derived from maternal tissue, all of the seed from a given tree will share megagametophyte genotype. This reduces genetic variability which is important to biochemical and molecular research. There is inherent genotypic variability in the embryo due to random pollen populations in plantations, but as mentioned above, this variability is usually restricted. Loblolly pine seed is an excellent system to direct studies of gene expression because of their industrial importance and the high degree of genetic uniformity in the megagametophyte tissue which can be attained.

1.3 SEED STRUCTURE

The mature seed of loblolly pine is typical of gymnosperms with the embryonic occupying a longitudinal position in a fluid-filled cavity at the center of the seed. Completely surrounding the embryo is the fleshy haploid female gametophyte (megagametophyte) which is formed prior to fertilization. The nucellus is reduced in the mature seed to a small cap-like structure covering the end of the

megagametophyte closest to the embryonic radicle, analogous to the position of the nucellus in western white pine (*Pinus monticola*) (Hoff 1987). Two distinct seed coat layers are formed from the integuments. The innermost integument forms a papery membrane that may act as a barrier to water adsorption (Baron 1978). The outermost layer of the seed coat is hardened and moderately thick at maturity. The embryo has 8 cotyledons, and greens immediately upon radicle emergence from the seed coat.

1.4 NUTRIENT RESERVE SYNTHESIS AND STORAGE

The period leading up to seed maturation is one of high metabolic activity. One of the major metabolic commitments at this time is the synthesis of reserves such as lipids, proteins and carbohydrates. Reserves play a very important role in seed germination and early seedling growth as will be discussed shortly. The synthesis of these reserves has been monitored in many species, and is under the direction of a set of genes active only at this time of development (Browder 1984, Higgins 1984). A significant portion of the reserves accumulated in gymnosperm seeds are lipids and proteins (Ching 1966, Baron 1970, Nyman 1971, Salmia 1981, Cardemil & Reinero 1982, Kovac & Wrischer 1984, Gifford 1988, Flinn *et al.* 1989, Gifford & Tolley 1989, Kovac & Kregar 1989).

The newly synthesized macromolecules in maturing seeds are sequestered in specialized tissues. In angiosperms,

reserves accumulate in the endosperm or cotyledons following fertilization. Gymnosperms are unique among seed plants in that the haploid megagametophyte is the primary nutritive tissue (Kovac & Wrischer 1984, Bewley & Black 1985, De Carli *et al.* 1987, Gifford & Tolley 1989, Cyr *et al.* in press). Unlike endosperm or cotyledons, megagametophytes are present prior to fertilization. The study of how this is regulated in conifers is therefore of great interest.

Within the nutritive tissue, reserves accumulate in specialized organelles. After synthesis on the rough endoplasmic reticulum, storage proteins are deposited in membrane enclosed protein bodies (Boulter 1981). The types of reserves found accumulated in the protein bodies differ between families in the plant kingdom. Protein reserves are classified according to their solubility characteristics in different solvents (Bewley & Black 1985). Dicots in general store primarily globulins as protein reserves in the seed; these are soluble in salt solutions (0.5-1.0 M NaCl) but not water. Monocot reserves, on the other hand, are dominated by prolamines (soluble in aqueous alcohols) and glutelins (soluble in dilute acid or alkaline solutions).

Limited attempts to qualify the protein reserves in gymnosperms have been made. Legumin-like reserves, members of the globulin class of proteins, have been documented to be the major storage proteins in *Ginkgo* seeds (Jensen & Berthold 1989). These proteins are very similar to angiosperm legumins in their solubility and structural

characteristics. A different type of protein reserve which is crystalloid in nature forms the majority of reserves in conifer genera including *Pinus* (Gifford 1988, Lammer & Gifford 1989), *Abies* (Green *et al.* 1991) and *Picea* (Flinn *et al.* 1991). Conflicting reports have emerged on the prevalence of crystalloid proteins in white spruce (*Picea glauca*) (Gifford & Tolley 1989, Misra & Green 1990). These proteins also occur in angiosperm dicots such as cucumber (*Cucumis sativus*) (Becker *et al.* 1978), and some members of the Euphorbiaceae (Gifford & Bewley 1983, 1984, Lalonde *et al.* 1984). Crystalloids are a class of globulins with a sedimentation coefficient of 11S which are insoluble in buffer solutions unless urea or SDS is added. In castor bean (*Ricinus communis*), the crystalloid holoprotein has a molecular mass of approximately 300 kDa comprised of six 50 kDa subunits, each subunit having a characteristic heterodimer structure (Gifford & Bewley 1983, 1984).

Like protein bodies, lipisomes are also synthesized from endoplasmic reticulum (Mayer & Marbach 1981). These single-membraned organelles enclose the lipid reserves within storage parenchyma cells. Lipid reserves often consist of a mixture of triglycerides, glycolipids, phospholipids, sterols and waxes. In conifers, reserves are typically triglycerides with a high linoleic acid composition (Kovac & Vardjan 1981, Flinn *et al.* 1989).

The period of reserve synthesis during seed development is terminated by seed desiccation, resulting in the fully

mature seed. The seed is then referred to as being quiescent, or rather, in a state in which germination can not occur until the appropriate environmental conditions are present. The drying out of the seed is essential for the suppression of the metabolic program involved in reserve synthesis. It is also a prerequisite to the switching on of germination-related metabolism (Kermode & Bewley 1986). Evidence suggesting that desiccation is involved in regulating this switch in gene expression at the level of protein synthesis has been shown in a number of genera including bean (*Phaseolus vulgaris*) (Dasgupta & Bewley 1982, Misra & Bewley 1985a), radish (Raphanus sativus) (Aspart *et al.* 1984), soybean (*Glycine max*) (Rosenberg & Rinne 1986) and castor bean (Kermode & Bewley 1985, Kermode *et al.* 1985). Kermode & Bewley (1986) have also shown that desiccation is necessary for the induction of specific proteins (enzymes) involved specifically in germination.

1.5 SEED GERMINATION AND RESERVE MOBILIZATION

The resumption of metabolic activity following quiescence in many seeds requires only imbibition with water and a supply of oxygen. The swelling of embryonic cells as a result of rehydration during seed imbibition in combination with the initiation of cell division in the radicle, creates a mechanical force, which splits the seed coat open (Mayer & Marbach 1981). Other seeds require additional triggers to initiate germination and are referred

to as being dormant. In many cases, it is the seed coat that imposes dormancy on the seed by preventing absorption of water or oxygen. Exposing the seed to conditions such as scarification (nicking of the seed coat), fire, or partial digestion of the seed coat by animal ingestion can overcome this dormancy. In seeds of lettuce (*Lactuca sativa* L.), activation of digestive enzymes such as esterases (Chandra & Toole 1977) and mannase (Halmer & Bewley 1979) are required to soften the endosperm and seed coat to allow the radicle to protrude. Many temperate tree species require a wet period accompanied by cold temperature in order to overcome dormancy. During such a treatment, germination stimulators develop (Stern 1991). This type of dormancy can be broken artificially in the lab by placing seeds under refrigerated damp conditions, a technique called stratification. Once the necessary environment for germination is created, new sets of genes are selectively turned on, providing some of the enzymes and other cellular components needed for germination and early seedling growth. The germinative period terminates with emergence of the radicle from the seed coat.

The switching from a quiescent to a dynamic state during imbibition also results in changes in both the ultrastructure and chemical composition of the seed. During early seedling growth, storage organelles (protein bodies and lipisomes) begin to disappear in the storage tissue. This change in ultrastructure is associated with hydrolysis

of protein and lipid reserves (Trelease *et al.* 1971, Simola 1974). Stored in relatively insoluble forms, the reserves must be converted to soluble compounds before they can be transported to the embryo where they are ultimately used as metabolic fuels and building blocks. Sugars resulting from lipid hydrolysis are transported to the embryo from the storage tissue in the form of sucrose (Murphy *et al.* 1991 in press), which is eventually metabolized to yield energy in the form of ATP and provide the critical requirements for many biosynthetic reactions during germination (Noland & Murphy 1984). Reserve proteins are converted to amino acids (Ching 1966) which are also translocated to the embryo. Amino acids can then be used as sources of energy or nitrogen for nucleotide and protein synthesis. The embryonic axis relies on these fuels and building blocks after its own reserves are depleted and before photosynthetic independence is attained (Ching 1972, Higgins 1984, Lammer & Gifford 1989).

Extensive studies on lipid and protein hydrolysis have shown strikingly similar patterns between plant species. The large quantities of lipids accumulated in the nutritive tissue of many angiosperm (Becker *et al.* 1978, Davies & Chapman 1979a, Weir *et al.* 1980) and gymnosperm seeds (Ching 1966, Durzan *et al.* 1971, Kovac & Wrischer 1984) are rapidly and constantly depleted following germination until reserves are almost completely exhausted. The rapid hydrolysis of storage proteins following radicle emergence has also been

shown in various species (Ching 1966, Davies & Chapman 1979a, Salmia 1981, Gifford *et al.* 1983, Kovac & Kregar 1989, Lammer & Gifford 1989, Gifford & Tolley 1989). Eventually as reserves are consumed in conifer megagametophytes, metabolic activity ceases and cell integrity is lost as a result of aging or senescing of the tissue (Simola 1974).

Depletion of the small amount of reserves in the embryo has received less attention. The hydrolysis of stored lipids and proteins is masked by the accumulation of these macromolecules during the rapid growth of the seedling. The hydrolysis of specific proteins in the embryo and nutritive tissue can be monitored using electrophoretic analysis. Following separation based on molecular mass in a porous gel by electrophoresis, proteins form a characteristic profile that can be seen by staining. When protein extracts taken at different stages of germination and early seedling growth are run on the same gel, decreases in the relative intensity of some of the protein bands can be detected. This is a good indication that these proteins are indeed storage reserves and are being hydrolyzed. Mobilization of major storage proteins has been monitored by this method in the megagametophytes of gymnosperms such as white spruce (*Picea glauca*) (Gifford & Tolley 1989) and in both the embryo and megagametophyte of lodgepole pine (*Pinus contorta*) (Lammer & Gifford 1989).

1.6 EVIDENCE OF THE REGULATION OF MOBILIZATION

The metabolic changes occurring during germination and early seedling growth must be precisely timed and regulated for the perpetuation of each species. Each viable seed is equipped with the necessary genetic information to ensure that the processes discussed above occur only under the right environmental conditions and in the proper sequence. Evidence that these processes reflect major alterations in gene expression has been shown in many plant species (Thompson & Lane 1980, Dure *et al.* 1981, Galau & Dure 1981, Aspart *et al.* 1984, Misra & Bewley 1985b, Sanchez-Martinez *et al.* 1986, Harada *et al.* 1988b).

Since the primary function of the genome is to direct protein synthesis, it is logical to asses whether or not protein synthesis is developmentally regulated prior to a study on gene expression. A general electrophoretic analysis of buffer-soluble proteins synthesized *de novo* following seed imbibition should reflect any changes in a tissue's capacity to synthesize proteins. Studies in a variety of species have given preliminary evidence of gene regulation by demonstrating changes in protein synthesis during germination and early seedling growth (Gientka-Rychter & Cherry 1968, Longo 1968, Cumming & Lane 1979, Dure *et al.* 1981, Marcus & Rodway 1982, Aspart *et al.* 1984, Rosenberg & Rinne 1989, Gifford *et al.* 1991).

Many specific proteins in plants are known to be synthesized only in a restricted phase of a life cycle, or in a particular tissue (Browder 1984). The enzymes involved

in the catabolic events of lipid and protein mobilization following the onset of germination are excellent examples of such proteins. The synthesis, activation, inhibition or degradation of these hydrolytic enzymes are all controlled and affect the amount of activity detected at any given time. Changing activities following germination have been studied in gymnosperm genera such as *Araucaria* (Cardemil & Reinero 1982), *Pinus* (Firenzuoli *et al.* 1968a, Ching 1970, Conkle 1971, Nyman 1971, Ramaiah *et al.* 1971, Bilderback 1974, Salmia & Mikola 1975, Salmia 1981, Noland & Murphy 1984, Pitel & Cheliak 1988, Gifford *et al.* 1989), *Abies* (Kovac & Wrischer 1984), *Larix* (Pitel & Cheliak 1986) and *Picea* (Gifford & Tolley 1989). Investigated in this thesis are two enzymes involved in lipid hydrolysis in loblolly pine, catalase and isocitrate lyase.

Many scientists are in agreement that the mature seed is supplied with preformed mRNAs synthesized during embryogenesis, which code for proteins synthesized early on in germination (Thompson & Lane 1980, Aspart *et al.* 1984). Ihle & Dure (1972) demonstrated the existence of preformed mRNA in cotton seed (*Gossypium* sp.), and were the first to suggest that some of these stored mRNA code for enzymes playing specific roles in germination, such as isocitrate lyase. Radin & Trelease (1976) and Smith *et al.* (1974) however, showed that transcription of new mRNA is necessary for the development of enzymes such as ICL. It is generally thought now that most enzymes involved in reserve hydrolysis

are synthesized from newly made, not preformed, mRNA (Rodriquez *et al.* 1987, Harada *et al.* 1988a).

1.7 THE ROLE OF CATALASE AND ISOCITRATE LYASE IN LIPID MOBILIZATION

Biochemical pathways which hydrolyze the reserves and allow transfer to the embryonic axis are essential for the conveyance of lipids stored in the megagametophyte of conifers to the embryo (Firenzuoli *et al.* 1968a, Ching 1970). Complete degradation of lipids occurs in two different organelles. Lipids are converted to fatty acids and glycerol in the lipisomes. The next steps involved in the process occur in the membrane-bound organelle, the glyoxysome (Trelease *et al.* 1971, Beevers 1979). Glyoxysomes were first discovered in gymnosperms by Ching (1970) working on ponderosa pine (*Pinus ponderosa* Laws). The oxidation of fatty acids into acetyl CoA (Mathews & vanHolde 1990) begins the breakdown process in the glyoxysome. Secondly, the resulting acetyl CoA is channelled into the glyoxylate cycle. During this critical cycle in fat-storing seeds, carbon derived from fatty acids is shunted towards the synthesis of carbohydrates while allowing regeneration of the components of the Kreb's cycle (Kornberg 1966).

The hydrogen peroxide produced during the oxidation of fatty acids in the glyoxysome is highly toxic to plants. Catalase (EC 1.11.1.6) is an essential enzyme in the glyoxysome which catalyzes the breakdown of 2 molecules of

H_2O_2 into 2 H_2O and O_2. Catalase also performs this function in peroxisomes later in seedling development. Isocitrate lyase (EC 4.1.3.1) is a key enzyme in the glyoxylate cycle, functioning in catalyzing the reversible cleavage of isocitrate into glyoxylate and succinate (first demonstrated in conifers by Firenzuoli *et al.* 1968b).

Miernyk *et al.* (1979) surveyed 19 different angiosperm oil seeds for the presence of ICL activity in mature seed. In all cases, ICL activity was absent. Inconsistencies exist in the literature on the presence of ICL activity in mature and stratified seed of various conifers. Italian stone pine (*Pinus pinea*) and ponderosa pine (*Pinus ponderosa*) had no detectable ICL activity (Firenzuoli *et al.* 1968, Ching 1970) prior to imbibition, while both Kovac & Wrischer (1984) and Noland & Murphy (1984) discerned low levels of activity in the mature megagametophytes of silver fir (*Abies alba* Mill.) and sugar pine (*Pinus lambertiana*). Catalase, on the other hand, is universally found in the nutritive tissue of mature seed in substantial levels (Choinski & Trelease 1978, Kovac & Wrischer 1984).

During early seedling growth, glyoxylate cycle enzymes undergo a well-characterized increase then subsequent decrease with peak activity corresponding to maximum lipid metabolism in the nutritive tissue (Carpenter & Beevers 1959, Ching 1970, Trelease *et al.* 1971, Kagawa *et al.* 1973, Bilderback 1974, Becker *et al.* 1978, Murray & Adams 1980, Kovac & Wrischer 1984). Catalase has been included in many

angiosperm studies dealing with glyoxylate cycle enzymes because of its similar kinetics, its presence in the glyoxysome, and its role in lipid mobilization. In cucumber, catalase activity is maintained in later stages of seedling growth in contrast to the rapid decrease in ICL activity (Becker *et al.* 1978). This is probably due to the additional function of catalase in peroxisomes. The changes in activity of enzymes involved in lipid mobilization appear to be regulated at the level of mRNA accumulation (Weir *et al.* 1980, Allen *et al.* 1988, Comai *et al.* 1989, Turley & Trelease 1990).

1.8 THE EMBRYO'S ROLE IN REGULATING MOBILIZATION OF RESERVES IN NUTRITIVE TISSUE

Because of the high degree of coordination between reserve hydrolysis in storage tissue and growth of the embryonic axis, the possibility of a regulatory interaction between the tissues has been studied extensively. In many species, evidence exists that the embryonic axis influences the regulation of reserve mobilization in the nutritive tissue of the seed. For example, removal of the axis prior to imbibition interferes with normal hydrolysis in the cotyledons of cucumber (Davies & Chapman 1979a,b, Slack *et al.* 1977), pea (*Pisum sativum* L.) (Chin *et al.* 1972), lentil (Alvarez *et al.* 1987) and bean (Kern & Chrispeels 1978, Minamikawa 1979, Morohashi 1982) and in the endosperm of castor bean (*Ricinus communis*) (Gifford *et al.* 1984). The

prerequisite of embryo attachment for normal reserve mobilization has also been shown for gymnosperm megagametophytes (Ching 1970, Nyman 1971). The decrease in hydrolysis can be attributed to a reduction in the specific activity of hydrolytic enzymes in pine (Bilderback 1974, Murray & Adams 1980), *R. communis* (Gifford *et al.* 1984), pea (*Pisum sativum*) (Guardiola & Sutcliffe 1971, Yomo & Varner 1973), bean (*Phaseolus vulgaris*) (Kern & Chrispeels 1978, Minamikawa 1979, Morohashi 1982), squash (*Cucurbita* sp.) (Penner & Ashton 1967), lentil (*Lens culinaris*) (Alvarez *et al.* 1987) and flax (*Linum* sp.) (Khan *et al.* 1976). However, a reduction in reserve hydrolysis due to embryonic axis removal does not seem to be correlated with lowered enzyme activity in a variety of other species; for example soybean (*Glycine max*) (Tester 1976), ground-nut (*Arachis hypogaea*) (Allfrey & Northcote 1977), cucumber (*Cucumis sativus*) (Slack *et al.* 1977, Davies & Chapman 1979a,b) and *P. vulgaris* (Tamura *et al.* 1982, Koshiba & Minamikawa 1983). In one isolated study on *R. communis*, removal of the embryo reduced the activity of enzymes involved in lipid hydrolysis, but no corresponding effect on lipid mobilization in the endosperm was seen (Huang & Beevers 1974).

The diverse results obtained from such experiments have evoked various explanations. Two mechanisms that attempt to explain the embryo's involvement in controlling these processes have been postulated in angiosperms. It is assumed that the embryonic axis exerts control over

mobilization of reserves in some monocot species by producing plant growth substances targeted to the nutritive tissue. For example, it is well documented that gibberellic acid secreted by the embryonic axis in barley (*Hordeum vulgare*) elicits the synthesis of α-amylase in the aleurone layer which in turn degrades starch reserves in the endosperm (Jacobsen & Varner 1967). The same dependency on the embryonic axis for some diffusible factor from the embryo has been shown in angiosperm dicots such as cucumber (Sze & Ashton 1971) and in conifers such as *P. ponderosa* (Bilderback 1974) and (*Pinus silvestris*) (Nyman 1971). The assumption is usually based on the observation that exogenously applied hormones can replace the axial requirement (Penner & Ashton 1967, Pinfield 1968, Doig *et al.* 1975, Halmer & Bewley 1979, Bewley & Black 1985, Pino *et al.* 1990). However, cases exist in both conifers and angiosperms where application of known hormones can not replace the requirement for the embryonic axis (Bilderback 1974, Mitsuhashi *et al.* 1984).

Hydrolysis in the food reserve may also dependant on the embryonic axis for continuous removal of end-products (Huang & Beevers 1974). If a product builds up to a certain level, an enzyme involved in the synthesis of the product may be inhibited. This type of controlling mechanism is termed feed-back inhibition and regulates many biological processes. For example, in pea, treatment of the bulky cotyledons with an excess of amino acids inhibits protease development (Yomo & Varner 1973). Davies & Chapman (1979b,

1980) have suggested feed-back inhibition as a regulatory mechanism in both protein and lipid hydrolysis of *C. sativus*.

1.9 THE PRESENT STUDY

This study was in part an attempt to elucidate the cellular changes occuring in loblolly pine seed during germination and early seedling growth, and to provide necessary background information needed to focus future effort on the regulation of specific genes during germination. Changes in lipid and protein reserves in the megagametophyte and embryo have been quantified. Shifts in protein patterns detected by one-dimensional electrophoresis were used to evaluate the depletion of specific protein reserves in the tissues. To show the correlation between reserve hydrolysis and product accumulation, quantitative changes in soluble amino acids were also investigated.

To further investigate developmental changes during the study period, shifts in protein synthesis, as seen by one- and two-dimensional electrophoresis, and corresponding changes in specific activities of catalase and isocitrate lyase were studied.

Finally, the degree of gametophyte dependance on the embryo for both reserve mobilization and the normal development of enzyme activities was examined. The effect of embryo removal on protein synthesis in the megagametophyte was also studied.

II. MATERIALS AND METHODS

2.1 CHEMICALS

Research grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Fisher Scientific (Edmonton, AB, Canada), and BDH Chemicals (Toronto, ON, Canada). Sigma also supplied pharmalytes, Rifampicin and Amphotericin B. [^{35}S]-labelled methionine, [^{14}C]-labelled methylated molecular weight markers and aqueous counting scintillant (ACS-II), were purchased from Amersham (Oakville, ON, Canada). En^{3}Hance was supplied by NEN Research Products (Boston, MA, USA). Bio-Rad Laboratories (Mississauga, ON, Canada) supplied non-radioactive molecular weight markers. Bacto-agar was obtained from Difco Laboratories (Detroit, MI, USA). Roccal was purchased from the National Laboratories (Toronto, ON, Canada). Unless otherwise stated, all water used in this study was distilled and deionized (conductivity $\geq$ 18 megohms).

2.2 EQUIPMENT

J2-21M/E Centrifuge, Microfuge E, LS 6000TA Scintillation counter and DU-65 Spectrophotometer were purchased from Beckman (Richmond, BC, Canada). From Bio-Rad Laboratories (Richmond, CA, USA), the gel dryer, mini-protean II dual slab cell and mini-protean II 2-D cell were obtained. Eppendorf E. Centrifuge 5415 was obtained from Mandel Scientific (Edmonton, AB, Canada). Kimpak was purchased from Seedboro Equipment (Chicago, IL, USA).

Germination trays were purchased from Spencer Lemaire Ind. (Edmonton, AB, Canada). Incubators used for stratification and germination were manufactured by Controlled Environments (Winnipeg, MB, Canada). Envirogard hood was purchased from EnVIROgineering Ltd. (Toronto, ON, Canada) and the fume hood was supplied by H.H. Hawkins Ltd. (Vancouver, BC, Canada).

2.3 SEED MATERIAL AND GERMINATION

Loblolly pine seed (*Pinus taeda* L.) was a gift from Westvaco, Summerville, SC, USA. The seeds were collected in 1984 from a first-generation select tree of loblolly pine (coded 11-9) which had been open pollinated. Prior to stratification, seeds were thoroughly sterilized according to Molina & Palmer (1982) as follows. Seeds were placed in a cheese cloth bag under running tap water for 30 min, followed by a 30 min shake in 0.02% Tween 20. The bag was rinsed under running tap water for 30 min, then shaken for 60 min in 30% H_2O_2. Seeds were rinsed in an Envirogard hood with two litres of autoclaved H_2O, removed from the cheese-cloth bag with sterile tweezers, and transfered between layers of moist Kimpak in an autoclaved germination tray (all autoclaving was for 20 min at 1.3 kg/cm2). Stratification was carried out at 4°C for 30 to 60 days in a dark incubator to overcome dormancy. For germination, seeds were removed from the germination tray, surface sterilized in 1% sodium hypochlorite for 1 min, rinsed thoroughly with sterile water, transferred to a moist Kimpak surface in a

freshly autoclaved tray, and imbibed at 30°C in an illuminated incubator (F-48-T-12 cool white fluorescent bulbs, 19 μmol m^{-2} sec^{-1}) for up to 12 days.

Germination was considered completed when the radicle emerged from the seed coat. Radicle size classes were determined for each day of imbibition at 30°C following radicle emergence. This was done by measuring the distance from the tip of the radicle to the point of emergence of the hypocotyl from the seed coat from 50 seeds. One standard deviation from the average was used as the radicle size class range.

2.4 EXPERIMENTS USING ISOLATED MEGAGAMETOPHYTES

Fully stratified seeds were surface sterilized and rinsed as above. Subsequent manipulation of the seeds was done in an Envirogard hood swabbed with ethanol and 0.4% Roccal for sterilization. Seed coats and nucelli were removed, and embryos (embryonic axis plus cotyledons) were excised by cutting lengthwise through the megagametophyte with an autoclaved sharp razor blade and removed with a sterile probe. Megagametophyte halves were placed in petri dishes containing 30 ml of sterile 3% bacto-agar containing 15 μg/ml of antibacterial Rifampicin (dissolved in 80% DMSO) and 2.5 μg/ml of antifungal Amphotericin B (dissolved in 75% DMSO). For amino acid exportation experiments, sectioned petri dishes containing 7.5 ml 0.8% bacto-agar (supplemented with bacterial and fungal inhibitors as above) in each

section were used. The megagametophytes were placed on the agar in one of two orientations: surfaces normally adjacent to the embryo were placed down on the agar, or, surfaces normally distal to the embryo were placed down. All petri dishes were placed in a large germination tray containing 50 ml sterile water in the bottom to maintain humidity. This large tray was then placed in an incubator at 30°C for up to 6 days.

2.5 LIPID ASSAY

Total lipids were extracted in methanol-chloroform according to Becker *et al.* (1978). One gram of tissue was ground in 5 ml of methanol-chloroform 2:1 (v/v) and centrifuged at 10 000 rpm (7 800 g) on a J2-21M/E Beckman centrifuge for 10 min at 20°C (Ching 1966). The supernatant was decanted and the pellet re-extracted by grinding again in 5 ml of methanol-chloroform. Supernatants from both were pooled in a separatory funnel, and an equal volume of 2 M KCl was added. After phase separation, the organic phase containing the lipids was drained into a preweighed beaker. The aqueous phase was then washed twice more with 3.5 ml of methanol-chloroform, collecting the organic phase in the beaker each time. The pooled organic phases were then evaporated to dryness overnight in a fume hood at room temperature, then placed in a 50°C oven until a constant weight was reached.

2.6 PROTEIN EXTRACTION AND DETERMINATION

All procedures for protein extractions were carried out at 4°C according to Gifford *et al.* (1982). Centrifugation was done on an Eppendorf microcentrifuge at 14 000 rpm (25 000 g). For mature dry seed, seed coats were removed after a 1 h imbibition in H_2O. Protein was extracted from isolated megagametophytes and embryos separated using sterile forceps and probe. Ten seed parts (10 megagametophytes or 10 embryos) were ground in 1 ml 0.05 M $NaPO_4$ (pH 7.0), in an ice-cold mortar and pestle, transferred to a microfuge tube, then centrifuged for 10 min. The buffer-soluble proteins in the supernatant were saved on ice for protein, isocitrate lyase, and amino acid assays, or treated with an equal volume of 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS (w/v) and 10% glycerol (v/v) (Laemmli 1970) and stored at -20°C for electrophoresis. The pellet was re-extracted 3 times and the supernatants discarded. The buffer-insoluble proteins in the pellet were then suspended in 0.5 ml extraction buffer, and solubilized by boiling for 5 min in an equal volume of Laemmli buffer. Samples were centrifuged for 10 min and the buffer-insoluble proteins in the supernatant were removed and kept on ice for protein assays, or stored at -20°C for electrophoresis. Protein was determined by the method of Lowry *et al.* (1951).

2.7 AMINO ACID ASSAY

Amino acids were quantified by the ninhydrin method of Rosen (1957) as follows. Buffer-soluble protein extracts were boiled for 2 min, and centrifuged on a Beckman E microcentrifuge for 10 min (10 000 rpm or 7 800 g). Concurrently 3 tubes for each assay containing 1 ml water and 1 ml 0.5 M $NaPO_4$ (pH 7.0) were brought to a boil. To each tube was added 50 µl of the boiled extract. One half ml of ninhydrin (3% in ethylene glycol monomethyl ether) and 0.5 ml AC/CN (0.2 mM Na cyanide in 2.6 M Na acetate pH 5.3) were added to 1 ml of the diluted extract. After boiling for 15 min, 5 ml 50% propan-2-ol was added. After cooling in the dark for 30 min at room temperature, the absorbance at 570 nm was read spectrophotometrically, and the amount of amino acids expressed as µmol equivalents of alanine.

To assay for amino acid exportation from megagametophyte halves into agar, tissue was discarded and the 0.8% agar was macerated and melted in a test tube. The melted solution was treated exactly as a boiled extract above. All amino acid assays were done in triplicate.

2.8 IN VIVO PROTEIN SYNTHESIS

Ten freshly harvested megagametophytes or embryos from whole seeds were labelled with 100 µCi (3.8 GBq) [^{35}S]-methionine (40 TBq/mmol) for 3 h. Preliminary studies on the effect of removal of the embryo on incorporation of [^{35}S]-methionine into megagametophytes showed a striking decrease in the tissue's ability to synthesize new proteins

(Table 1). Because of this problem, 40 megagametophyte halves imbibed previously with or without the embryo were labelled with 200 μCi (7.6 GBq) [^{35}S]-methionine (40 TBq/mmol) for 3 h to insure enough incorporation for electrophoretic analysis. Tissue was then rinsed with H_2O and buffer-soluble proteins were extracted at 4°C in 1 ml 50 mM $NaPO_4$ (pH 7.5) containing 0.1 mM leupeptin as a protease inhibitor. Radioactivity was determined by trichloroacetic acid precipitation (Gifford & Bewley 1984) as follows. Ten μl of labelled extract was spotted on a 2.4 cm Whatman GF/C filter and washed under vacuum with 3 X 10 ml ice cold 10% TCA. Filters were then boiled for 15 min in 1 ml 10% TCA. Subsequent washes included 2 X 10 ml ice cold 10% TCA, and 4 X 2.5 ml ice cold EtOH. After drying, filters were placed in 10 ml ASC II and radiolabel incorporation determined by scintillation counting.

2.9 ELECTROPHORESIS

2.9.1 One-dimensional

Single dimension SDS-PAGE as described in Gifford & Bewley (1983) was carried out under reducing (+ME) and non-reducing (-ME) conditions on a Bio-Rad Laboratories mini gel system, using a 0.75 mm 12% acrylamide slab gel. Samples were equally loaded either on the basis of TCA-precipitable radioactivity, or on μg of protein. Prior to electrophoresis, some extracts were reduced with 2% ME to disrupt protein-protein disulphide bridging.

2.9.2 Two-dimensional

Isoelectric focusing (IEF) was carried out on a Bio-Rad Laboratories mini tube gel system. Samples treated with 9.5 M urea were loaded at the cathode in a tube gel containing 9.2 M urea, 4% acrylamide, 1.6% 5/8 pharmalyte and 0.4% 3/10 pharmalyte. Samples were then separated in the first dimension by charge (750 V). The cathodic solution used was 20 mM NaOH and the anodic solution was 10 mM H_3PO_4. Proteins were then separated in the second dimension on a 12% acrylamide slab gel according to molecular mass by SDS-PAGE as above.

2.9.3 Staining and fluorography

Following electrophoresis, non-labelled protein gels were stained with Coomassie blue R (Burk *et al.* 1983) and dried at 80°C for 2 h on a gel dryer. Molecular weight of proteins was determined by the method of Weber & Osborne (1969). Molecular weight markers included: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2; ovalbumin, 45.0; carbonic anhydrase, 31.0; soybean trypsin inhibitor, 21.5; lysozyme, 14.4. Some gel profiles were quantified by densitometry at 560 nm using a spectrophotometer.

Labelled proteins were enhanced for autoradiography and fixed into gels as follows. Gels were shaken for 40 min in 100 ml En^3Hance (used), then 40 min in 100 ml new En^3Hance. The gels were then shaken in H_2O for 30 min, dried under

vacuum at 60°C for 2 h on a gel dryer, and exposed to x-ray film (X-Omat AR-5) for autoradiography at -70°C (Gifford & Bewley 1984). [^{14}C]-methylated molecular weight markers for labelled gels were: myosin, 200 kDa; phosphorylase b, 92.5; bovine serum albumin, 69.0; ovalbumin, 46.0; carbonic anhydrase, 30.0; lysozyme, 14.3.

2.10 ENZYME ASSAYS

2.10.1 Catalase assay

Catalase was extracted from megagametophytes and embryos in the same manner as buffer-soluble proteins; using 0.05 M KPO_4 pH 7.5 as the extraction buffer. The optimum buffer pH was predetermined by varying the pH of buffer used and assaying as follows. All CAT assays were done in triplicate. The CAT kinetic assay contained 0.135 M KPO_4 pH 7.0, 0.096% H_2O_2, 0.0125% Triton X-100, and 100 µl of the extract in a final volume of 3.0 ml (Luck 1963). The first order degradation of H_2O_2 was monitored spectrophotometrically as a change in absorbance at 240 nm after blanking with all solutions except protein. One unit of CAT activity was that amount of enzyme required to degrade 50% of the H_2O_2 in 1 h at 25°C. Enzyme activity was expressed as units per seed part and units per µg protein.

2.10.2 Isocitrate Lyase Assay

The ICL kinetic assay was done in triplicate, and contained 50 mM $NaPO_4$ pH 6.0, 1.4 mM $MgCl_2$, 1.62 mM

phenylhydrazine hydrochloride (made fresh daily), 1.1 mM DL-dithiothreitol, 1.0 mM DL-isocitric acid, and 0.1 ml buffer-soluble protein extract in a final volume of 2.5 ml (Lamb *et al.* 1978). The optimum buffer pH was predetermined by varying the pH of buffer used and assaying as follows. The formation of glyoxylate phenylhydrazone was monitored spectrophotometrically as a change in absorbance at 324 nm after blanking with the assay mixture minus the protein extract. One unit of ICL activity was that amount of enzyme required to degrade 1 nmol of DL-isocitrate to glyoxylate and succinate per h at 25°C in the assay volume. Enzyme activity was expressed as units per seed part and units per μg protein.

III. RESULTS

3.1 WHOLE SEED

3.1.1 Seed stratification, germination and radicle growth

The requirement for a stratification period in order to overcome seed dormancy in loblolly pine is depicted in Figure 1. Only 20% of the seed germinated prior to stratification, but following 45 to 60 days of stratification at 4°C, germination increased to 90%. Relative water content of whole seed rose from 13.5 ± 0.4% to 30.4 ± 0.1% during this period. After transferring seeds to an incubator set at 30°C, completion of germination, as defined by radicle emergence from the seed coat, occurred at 4 DAI (days after imbibition at 30°C). Subsequent growth was defined as early seedling growth. Radicle lengths (hypocotyl plus radicle) during early seedling growth were measured daily. The relationship between mean radicle length of whole loblolly pine seeds and DAI is illustrated in Figure 2. Distinct radicle size classes for each day of imbibition following germination were chosen to reduce the variability in harvested material, and are tabulated in Figure 2. By 12 DAI, visible decay of the megagametophyte occurred accompanied by a decline in metabolic activity (senescence).

3.1.2 Reserve hydrolysis

3.1.2.1 *Quantitative changes in total lipid reserves*

The lipid content of the megagametophyte and embryo of mature loblolly pine seed on a fresh weight basis was 20% and 26% respectively (Table 2). The lipid content of the megagametophyte, however, accounted for 87% of the total lipid reserves of the mature seed. Quantitative changes in lipid reserves of both tissues from whole seed are shown in Figure 3. During germination, little of the lipid reserve of the megagametophyte was depleted until just prior to radicle emergence. As germination was completed and early seedling growth advanced, the reserves were nearly exhausted. By 12 DAI, total lipid fell from 20% to 4% of the megagametophyte's fresh weight, a loss of approximately 2 mg.

The amount of total lipid in the embryo rose slowly following imbibition at 30°C, and then fell slightly following radicle emergence. After 6 DAI, lipids increased from 0.23 mg to 1.64 mg (about 4.5 times the level in the mature seed). However, it should be noted that embryo fresh weight also increased during this period. Thus, the percentage of lipid on a fresh weight basis did not increase but fell from 26% to 3%.

3.1.2.2 *Quantitative changes in total protein storage reserves*

The megagametophyte stores 89% of the mature seed's protein, 75% of which is insoluble in buffer (Table 2). Buffer-insoluble proteins in the megagametophyte were

depleted at a constant rate, beginning before radicle emergence (Figure 4A). Levels by 12 DAI were one-tenth of dry seed reserves. An increase in the pool of soluble proteins in the megagametophyte did not accompany the initial decrease in the insoluble fraction (Figure 4A). The levels per seed remained relatively constant until 5 DAI, after which an increase occurred to reach two-fold by 9 DAI. This soluble pool was rapidly depleted thereafter as the tissue started to senesce.

Protein changes in the embryo were unlike those found in the megagametophyte (Figure 4B). Accumulation of both the soluble fraction (comprising 65% of the total embryo protein, Table 2) and the insoluble fraction were slow for the first 6 days of imbibition, but was followed by a rapid surge. At 9 DAI, the two fractions were equal in abundance. While soluble proteins continued to rise after 9 DAI, the insoluble fraction remained constant.

3.1.2.3 *Quantitative changes in soluble amino acid content*

A significant rise in the pool of soluble amino acids in the megagametophyte did not accompany the hydrolysis of storage protein reserves in this tissue (Figure 5). A rise of only 3 μmol equivalents of alanine occurred by 9 DAI, and levels decreased slightly thereafter. The embryo, on the other hand, showed a prominent and rapid increase in soluble amino acids during early seedling growth of almost 20 times (18 μmol). Embryos were excised from the megagametophytes

and grown *in vitro* on agar to test whether the increase in amino acids in the embryo could be reproduced without contact between the two tissues. A negligible increase in amino acids was detected under these circumstances.

3.1.3 The seed proteins

3.1.3.1 *Seed protein profiles*

Figure 6 shows soluble and insoluble protein fractions from the megagametophyte and embryo of fully stratified seed, electrophoretically separated under reducing and non-reducing conditions. The profiles of embryo and megagametophyte proteins were very similar under both conditions. For example, a 60 kDa band (apparent in lanes MI & EI of Figure 6A) upon treatment with ME disappeared from the profiles while two polypeptides with molecular masses of 37.5 and 22.5 kDa in both tissues, (lane MI & EI of Figure 6B) which were not present prior to reduction, appeared. A 47 kDa insoluble protein with solubility characteristics similar to the 60 kDa protein was not affected by ME treatment in either the embryo or the megagametophyte. Soluble proteins in the megagametophyte and embryo were similar, with major bands corresponding to molecular masses of 32 and 24-26 kDa which were not affected by ME treatment.

3.1.3.2 *Changes in profiles during imbibition*

The insoluble protein profiles of the megagametophyte and embryo were followed throughout germination and early seedling growth by one-dimensional electrophoresis, and visualized by Coomassie blue staining (Figures 7 & 8). During early seedling growth in the megagametophyte, a visible decrease in the 60 and 47 kDa bands was manifest (Figure 7A). Commencing at 7 DAI, there was a marked increase in relative intensity of two polypeptides with molecular masses of 37 and 17 kDa. The changes observed for the 47 and 17 kDa proteins were not affected by ME treatment (Figure 6B). The two proteins which replace the 60 kDa protein upon reduction with ME (37.5 & 22.5 kDa) mirrored the hydrolysis of the larger heterodimer. The 37 kDa protein in Figure 7A was also absent in reduced profiles, being replaced by two lower molecular mass proteins (18 & 21 kDa) which increased in abundance in the same fashion as the 37 kDa protein. Decline of the 22.5 kDa protein in Figure 7B was partially obscured by the increase in the slightly lower molecular mass 21 kDa polypeptide immediately below it.

The changes occurring initially in embryo insoluble protein profiles were similar in nature to those seen in the megagametophyte. The relative intensity of the 47 kDa band (Figure 8A) rapidly decreased, along with the 60 kDa protein and its corresponding subunit polypeptides upon reduction in Figure 8B. Of major difference between the embryo and the

megagametophyte was the absence of any polypeptides appearing at approximately 7 DAI and increasing thereafter.

In contrast to the insoluble proteins, the soluble protein profiles showed limited changes during germination and early seedling growth (Figure 9). The most noticeable change in the megagametophyte was the rapid hydrolysis of a 32 kDa protein (Figure 9A). This was accompanied by a diminishing amount of polypeptides in the 15 to 35 kDa range, and an accumulation of very low molecular mass proteins. The embryo showed an even more pronounced depletion of proteins in the 15 to 35 kDa range (Figure 9B). At 7 DAI, increases in two embryonic proteins corresponding to molecular masses of 53 and 14.5 kDa commenced.

3.1.3.3 *Scanning densitometry*

Relative levels of specific insoluble proteins were measured by scanning densitometry and plotted in Figure 10. The depletion of the 47 kDa protein in the megagametophyte reflected the quantitative changes occurring in the total insoluble fraction from that tissue (refer to Figure 4). The 37.5 kDa reduced protein in the megagametophyte was also mobilized quickly. Proteins of the same molecular masses in the embryo showed similar patterns of depletion.

Scanning densitometry was also used to monitor the increase in relative intensity of specific proteins appearing later on in seedling growth. The depletion of the large 60 kDa megagametophyte protein found in the mature

seed was accompanied by a corresponding rate of increase in two insoluble proteins of molecular masses 37 and 17 kDa. The inverse correlation between the 60 kDa reserve protein and the 37 kDa insoluble protein is shown in Figure 11. Curves were obtained using a second order regression analysis. As a result of other bands interfering with accurate scanning of the 60 kDa band, its hydrolysis was represented by its 37.5 kDa subunit polypeptide.

3.1.4 In vivo protein synthesis

3.1.4.1 *One-dimensional electrophoresis*

Protein synthesis in the megagametophyte was primarily restricted to polypeptides between 35 and 100 kDa (Figure 12), with only subtle transitions occurring during germination and early seedling growth. The most significant change was the synthesis of a 41 kDa protein at 4 DAI which increased in intensity with time. There also appeared to be a slight shift in protein synthesis during stratification. A reduction in the number of proteins synthesized in the embryo during the period of stratification was most apparent in the 14 to 40 kDa range. Synthesis of a major 53 kDa protein increased dramatically during early seedling growth. The only other detectable change was a slight increase in a low molecular mass protein (14.5 kDa) following germination. Changes in relative amounts of specific proteins were more evident when viewed by two-dimensional analysis.

3.1.4.2 *Two-dimensional electrophoresis*

Two-dimensional electrophoretic analysis of lobolly pine distinguished between the proteins synthesized in the megagametophyte and embryo of mature seed (Figures 13 & 14). While the embryo was very active in incorporating label, the megagametophyte was relatively idle. During stratification a shift in protein synthesis occurred. A distinct set of proteins synthesized in the fully stratified seed and at all subsequent stages was noticeably absent in the mature seed. The synthesis of one unique embryonic protein (indicated by open triangle) was detected in the mature seed. Likewise, the mature megagametophyte synthesized one low molecular mass protein that was not present at any other stage.

Gels of fully stratified seed revealed that most newly synthesized proteins at this stage have molecular masses between 40 and 100 kDa (Figures 13 & 14). Only a few low molecular mass proteins were synthesized in the megagametophyte, and even fewer in the embryo. Although the soluble protein profiles in the two tissues were very similar as seen in Figure 8, the synthesis of new proteins was distinct.

While most proteins were synthesized constitutively following stratification, many factors influenced their relative abundance. For example, two megagametophytic proteins corresponding to a molecular mass of 17 kDa declined gradually with time (Figure 13). In contrast, the relative abundance of a 53 kDa embryonic protein present at

every stage studied increased in abundance with time (Figure 14).

Proteins with more restricted temporal regulation of synthesis occurred in both tissues. In the embryo there were two low molecular weight proteins (within large boxes in Figure 14) which were only synthesized after radicle emergence. No proteins in the megagametophyte fit into this post-germinative category. However, a subset of proteins synthesized only during germination, and not after radicle emergence, was apparent in both tissues (circled in Figures 13 & 14). With one exception, the megagametophytic proteins falling into this category were of low molecular weight. One protein (circled in Figure 14) in the embryo was synthesized during germination exclusively, corresponding to a molecular mass of 55.5 kDa.

3.1.5 Enzyme activities

3.1.5.1 *Effect of pH on enzyme activities*

Curves in Figure 15 were obtained by second order regression analysis. The pH optimum for catalase in whole germinated loblolly pine seed using a 0.05 M KPO_4 buffer was found to be 7.0 (Figure 15A). The optimum pH for the 0.05 M $NaPO_4$ buffer used in the isocitrate lyase assay was 6.5 (Figure 15B).

3.1.5.2 *Changes in catalase activity*

Mature seed had detectable, but low levels of catalase activity in both the embryo and the megagametophyte. Activity rose slightly in the megagametophyte during stratification. Elucidation of the changes in catalase activity upon imbibition showed that activity increased slowly in the megagametophyte during germination, and rose rapidly (on a seed part basis) once the radicle emerged (Figure 16A). Activity remained constant after peaking at 7 DAI, and fell as the tissue senesced. The embryo had a much lower activity than the megagametophyte at all stages when compared on either a per seed part basis or on a total protein basis (Figures 16A & B). Maximal activity occurred on a per seed part basis at 9 DAI, and was highest in terms of protein content at 6 DAI.

3.1.5.3 *Changes in isocitrate lyase activity*

ICL activity was undetected in mature loblolly seed regardless of the tissue assayed, however, low activity was found in the megagametophyte following the stratification period. ICL activity in the megagametophyte was considerably slower than catalase in building to maximum levels (Figure 17A & B) upon imbibition. There was a large surge in specific activity at 7 DAI with the highest activity occurring at 9 DAI (or 11 DAI based on protein content) followed by an immediate plunge. Activity in the embryo was undetectable until 3 DAI, and reached only one

fifth of the activity in the megagametophyte regardless of how the data were expressed.

3.2 THE ROLE OF THE EMBRYO IN CONTROLLING MEGAGAMETOPHYTIC PROCESSES

3.2.1 Radicle growth

Table 3 shows changes in radicle lengths of seeds imbibed at 30°C on agar which had their seed coats and nucelli removed. Seeds treated in this manner are referred to as coatless seeds in this thesis. Removal of the seed coat as a barrier to germination accelerated radicle growth. By 1 day after imbibition radicles were already protruding from the megagametophyte. This table also compares radicle lengths of coatless seeds to the closest corresponding whole seed DAI (based on radicle size classes) to assist in comparing the two sets of data. In general, growth in coatless seeds was advanced 3 DAI ahead of whole seeds. Coatless intact seeds served as controls for the remaining experiments.

3.2.2 Effect of the embryo on reserve hydrolysis

3.2.2.1 *Quantitative changes in total lipid reserves*

Figure 18 shows that mobilization of lipid reserves was not inhibited by excision of the embryo. During 5 days of imbibition with embryos intact, a loss of 1.5 mg of lipid occurred. Removal of the embryo had no effect on this net lipid hydrolysis in the megagametophyte halves regardless of

their orientation in the agar. Embryo removal did, however, cause a more rapid initial drop in lipid reserves.

3.2.2.2 *Quantitative changes in total protein storage reserves*

Within 5 days following germination, megagametophytes incubated with the embryo intact lost 650 µg of insoluble protein reserves. Removal of the embryo had no significant effect on the rate of this hydrolysis of protein regardless of the orientation of the megagametophyte halves on the agar (Figure 19).

3.2.2.3 *Quantitative changes in soluble amino acid content*

Embryo attachment was not a prerequisite for the exportation of amino acids from the megagametophyte. By simply replacing the sink effect of the embryo with agar, there was a continual movement of the products of hydrolysis from the megagametophyte halves in concert with reserve hydrolysis (Figure 20). The amount of exportation measured did not, however, account for the high accumulation of amino acids in the intact embryo in Figure 4. At 11 DAI, 5 µmol equivalents of alanine were exported into the agar. At the same stage of development, isolated embryos contained only 1 µmol, accounting for only 6 of the 14 µmol equivalents of alanine accumulated by 11 DAI in embryos grown in contact with the megagametophyte.

3.2.2.4 *Scanning densitometry of specific proteins*

The effect of the embryo on hydrolysis of the 60 kDa buffer-insoluble protein was monitored by subjecting protein extracts from different stages after embryo removal to one-dimensional electrophoresis. The rate of hydrolysis of the 37.5 kDa subunit polypeptide was unaffected by removal of the embryo prior to imbibition (Figure 21). By 6 DAI, the percent area occupied by this protein as indicated by scanning densitometry decreased approximately 40% in both cases. Neither was there a substantial difference in the accumulation of the 37 kDa product. The mobilization pattern was comparable to that obtained for whole seed (Figure 11), though not identical.

3.2.3 Effect of the embryo on in vivo protein synthesis

Removal of the embryo prior to imbibition caused a dramatic and rapid switch in protein synthesis in the megagametophyte as seen in the fluorographs of Figure 22. Almost 50% of the proteins synthesized constitutively were either synthesized to a greater (representative proteins are boxed) or to a lesser (representative proteins are circled) extent when the embryo was removed. These proteins occurred in a wide range of molecular masses with noticeable trends in isoelectric points. Proteins with synthesis that was intensified by embryo removal tended to have isoelectric points more neutral to basic, while proteins synthesized to a lesser degree appeared more in the neutral to acidic

region. The proteins indicated with arrows were either synthesized only in the presence of the embryo, or only in the absence. After only 12 h of imbibition without the embryonic axis, one unique protein with a molecular mass of approximately 100 kDa was synthesized. Twelve hours later, another protein (43 kDa) only synthesized in the absence of the embryo, was evident. Following radicle emergence (2 DAI) a third protein restricted to those megagametophytes incubated without the embryo with a molecular mass of 15 kDa appeared. The synthesis of 2 proteins with molecular masses of 180 and 69 kDa was dependant on the presence of the embryo during imbibition.

3.2.4 Effect of the embryo on enzyme activities

3.2.4.1 *Changes in catalase activity*

When coatless seeds were incubated at 30°C for up to 6 DAI on agar, catalase activity in the megagametophyte increased much like that for megagametophytes from whole seeds (compare Figures 23A & 16). Keeping in mind the three day acceleration of growth in coatless seeds, both sets of data showed congruent and simultaneous optimum catalase activities. After germination in coatless seeds, there was a rapid tenfold increase in activity over 3 days, followed by a slight decline. When embryos were removed prior to imbibition and megagametophyte halves were placed with the surface usually proximal to the embryo down, enzyme activity was reduced significantly to one quarter of the specific

activity of intact seeds (55 units). However, when megagametophyte halves were placed with the surface usually distal to the embryo down, activity increased more rapidly than in megagametophytes which were imbibed with embryos intact, but this activity was not sustained for as long, nor did it reach the level that intact seeds did.

3.2.4.2 *Changes in isocitrate lyase activity*

There was an analogous development of ICL activity with respect to catalase in coatless seeds (Figure 23B). After germination there was a 10 fold increase, with maximum activity occurring at 4 DAI, and a subsequent reduction in activity. When megagametophyte halves imbibed without embryos were placed with the surface usually adjacent to the embryo down on agar, specific activity was reduced to less than half that of the intact seeds (675 units), with no definite peak developing. When placed with surfaces usually distal to the embryo down, ICL activity was even more markedly reduced and fell rapidly after 3 DAI to undetectable levels by 5 DAI.

Table 1 Effect of the embryo on incorporation of [^{35}S] methionine into megagametophytes.

DAI	Incorporation (dpm per megagametophyte)* imbibed with embryo	imbibed without embryo
1	428 810	447 130
2	267 085	19 130
3	305 615	18 590
4	249 995	18 195
5	152 140	7 520
6	62 600	3 885

*in each case 100 μCi (3.8 GBq) of label (40 TBq/mmol) was added to 20 megagametophyte halves for 3 h following imbibition for the designated time period at 30°C.

Table 2 Protein and lipid content of the megagametophyte and embryonic axis of mature seed.

Seed part	Seed part weight	Protein/seed part*						Total lipid/ seed part**	
		Total		Insoluble		Soluble			
			% of seed		% of total		% of total		% of seed
	mg	mg	part wt.	mg	mg	mg	mg	mg	part wt.
gametophyte	11.11	1.706 ± 0.2	15	1.262 ± 0.1	75	0.443 ± 0.2	25	2.185 ± 0.2	20
embryo	1.25	0.213 ± 0.05	17	0.074 ± 0.03	35	0.138 ± 0.03	65	0.330 ± 0.1	26

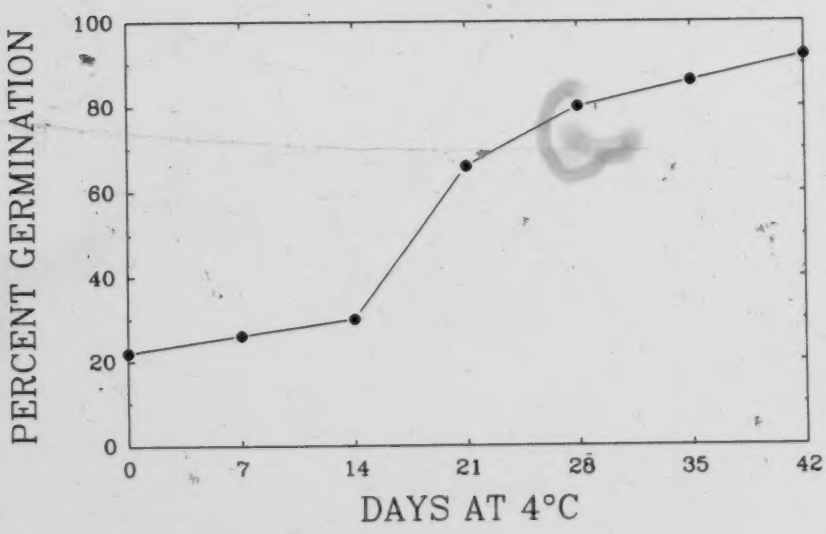
* values are a mean of 3 determinations ± SD.
** values are a mean of 2 determinations ± SD.

Table 3 Changes in radicle lengths of seeds with seed coats removed following imbibition at 30°C and the closest corresponding DAI based upon radicle size classes from whole seed data (see Figure 1).

DAI	Radicle length (mm)* mean	SD	range	Closest corresponding DAI based upon radicle size class
1	0.0	0.0	0	4 DAI
2	3.2	2.0	1.2-5.2	5 DAI
3	4.3	2.8	3-6	6 DAI
4	10.8	6.7	8-14	7 DAI
5	21.1	11.2	16-27	8 DAI
6	40.0	16.3	32-48	9-10 DAI

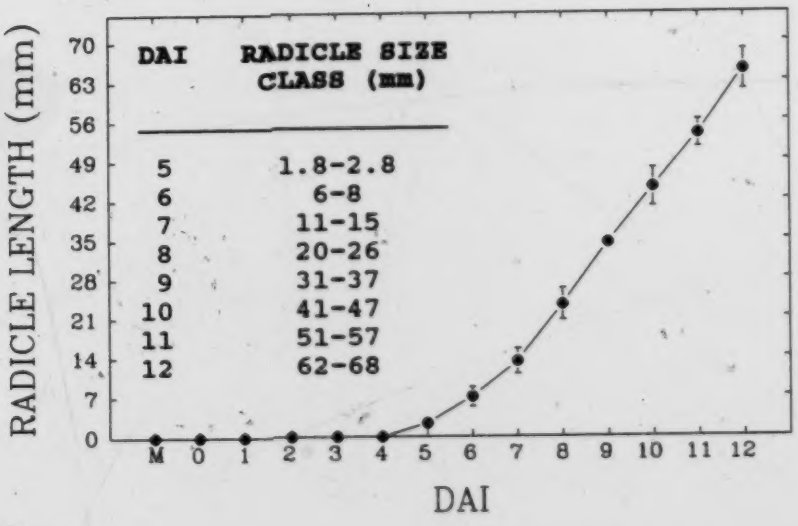
*in each case, radicles from 50 seeds were measured

Figure 1 **The effect of stratification period on percent germination.**



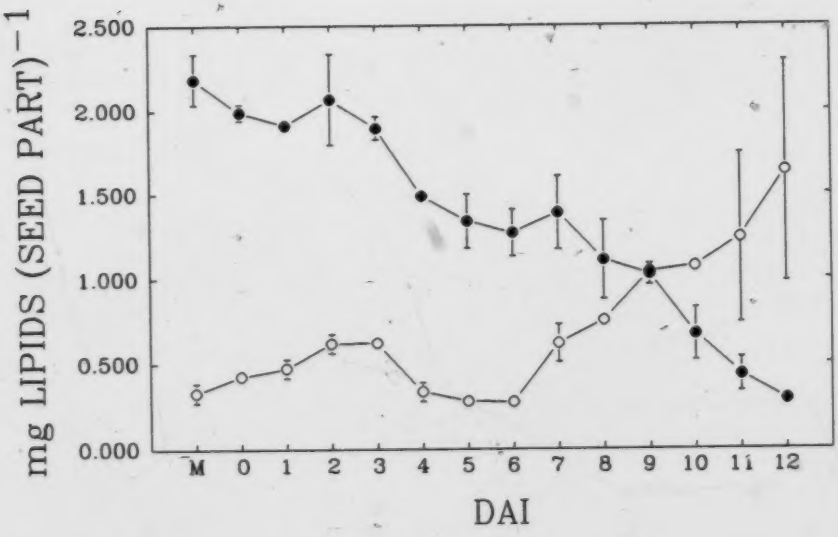
Each point is a mean of 150 determinations.

Figure 2 Changes in radicle lengths following seed imbibition at 30°C and designated post-germinative radicle size classes.



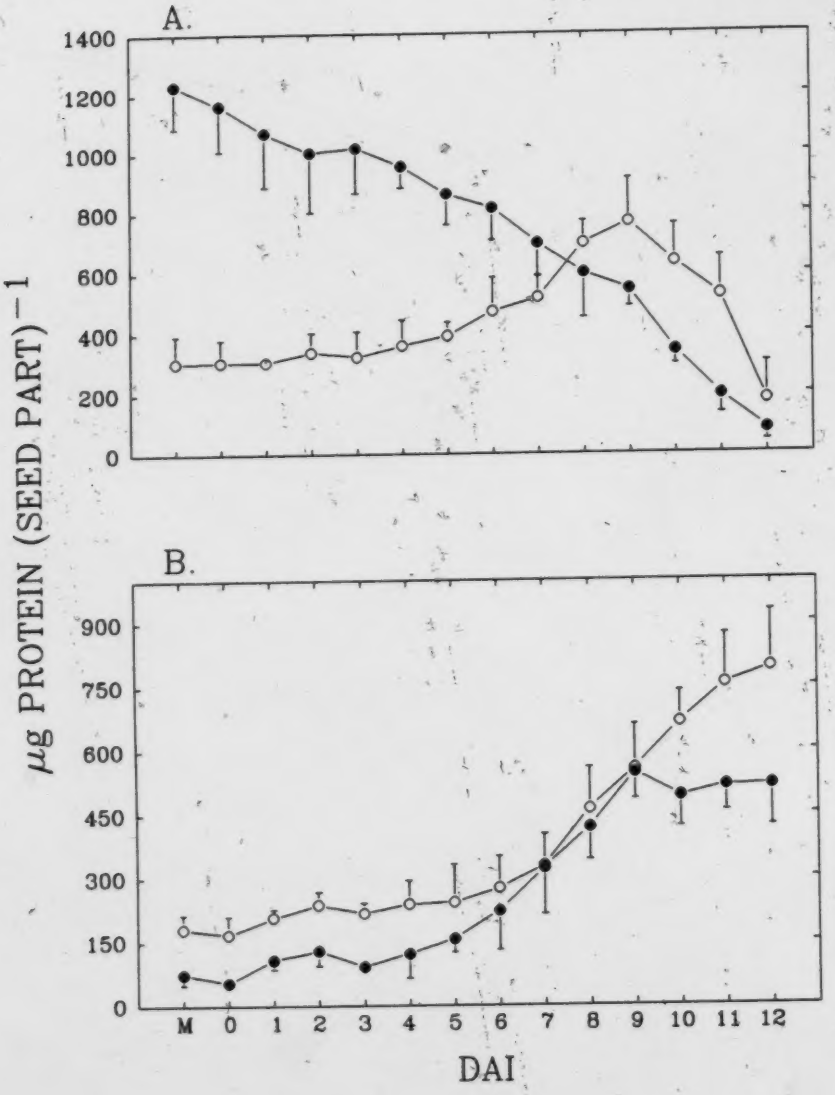
Each data point is a mean of 100 determinations ± SD. M, mature seed; 0, fully stratified seed; 1-12, days after imbibition at 30°C (DAI).

Figure 3 Quantitative changes in total lipids in the megagametophyte and embryo from whole seed following imbibition.



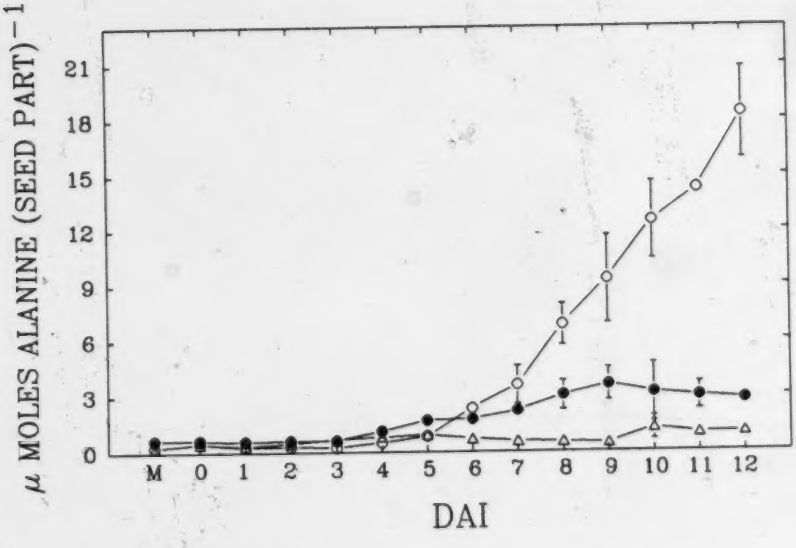
●, megagametophyte; o, embryo. Labels as for Figure 2. Each data point is a mean of 2 determinations ± SD.

Figure 4 **Quantitative changes in soluble and insoluble proteins of the megagametophyte and embryo from whole seed following imbibition.**



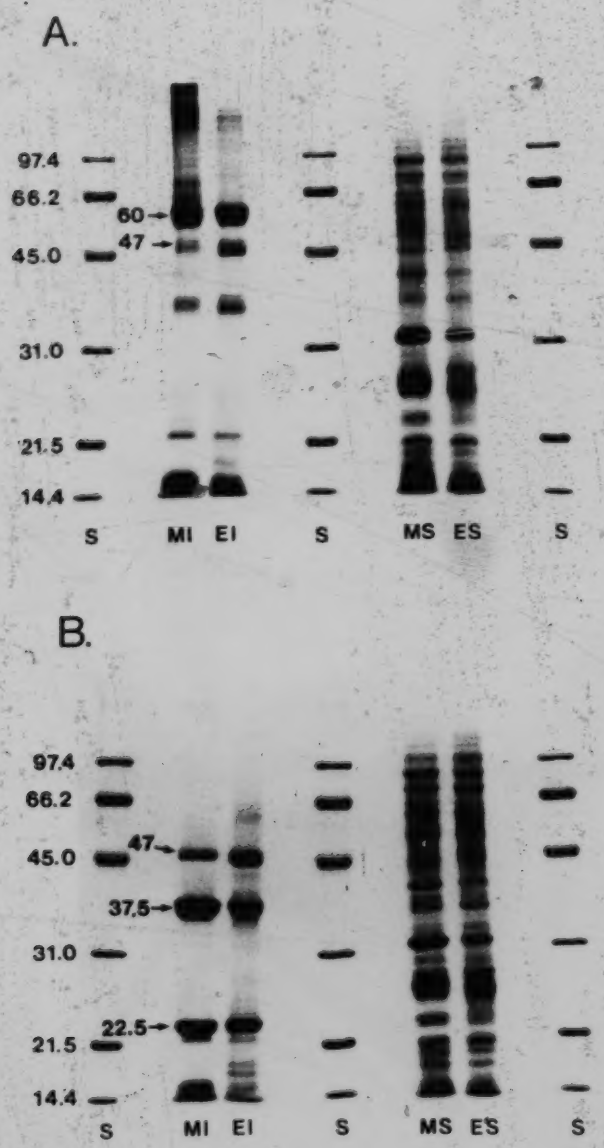
A, megagametophyte; B, embryo; o, soluble protein; ●, insoluble protein. Each data point is a mean of 3 determinations ± SD. Labels as for Figure 2.

Figure 5 **Changes in soluble amino acid content of the megagametophyte and embryo following seed imbibition.**



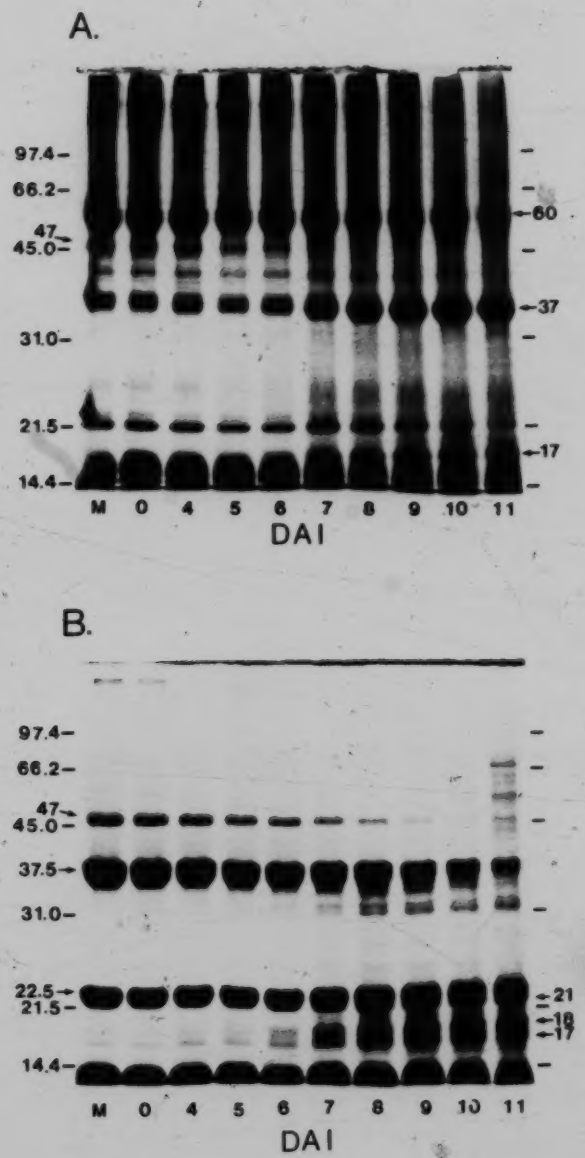
o, embryo; ●, megagametophyte; △, embryos grown *in vitro*. Each data point is a mean of 3 determinations ± SD. Labels as for Figure 2.

Figure 6 Coomassie stained SDS-PAGE profiles of soluble and insoluble fractions of the megagametophyte and embryo of fully stratified seed.



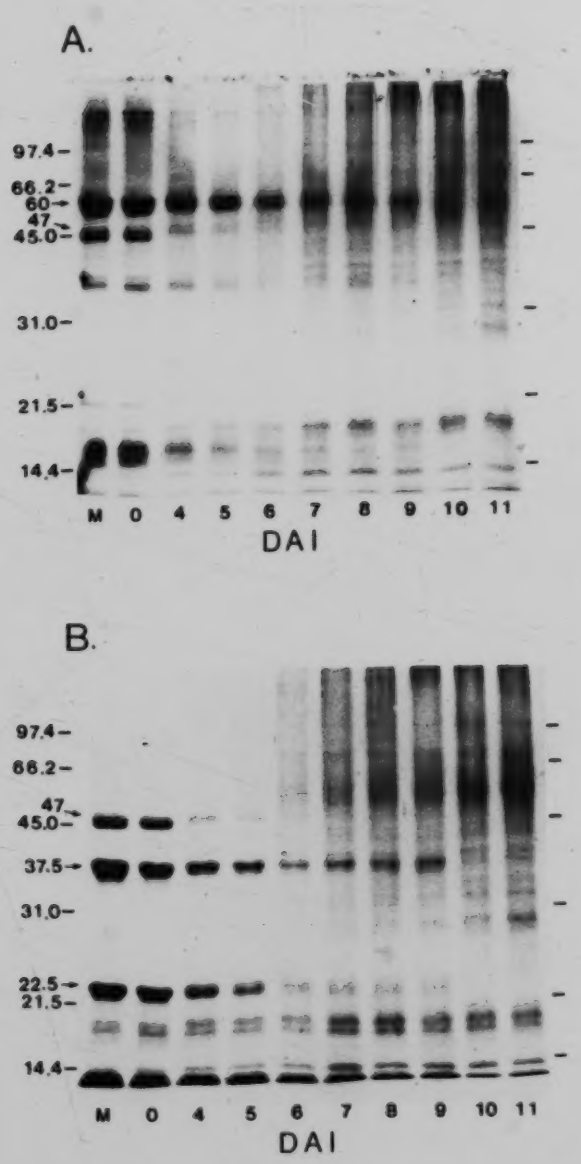
A, non-reduced; B, reduced; S, molecular weight standards with corresponding weights in kDa adjacent to gels; MI, megagametophyte insoluble proteins; EI, embryo insoluble proteins; MS, megagametophyte soluble proteins; ES, embryo soluble proteins. Arrows indicate significant proteins with their corresponding molecular masses.

Figure 7 **Coomassie stained SDS-PAGE profiles of insoluble protein fractions from the megagametophyte following imbibition.**



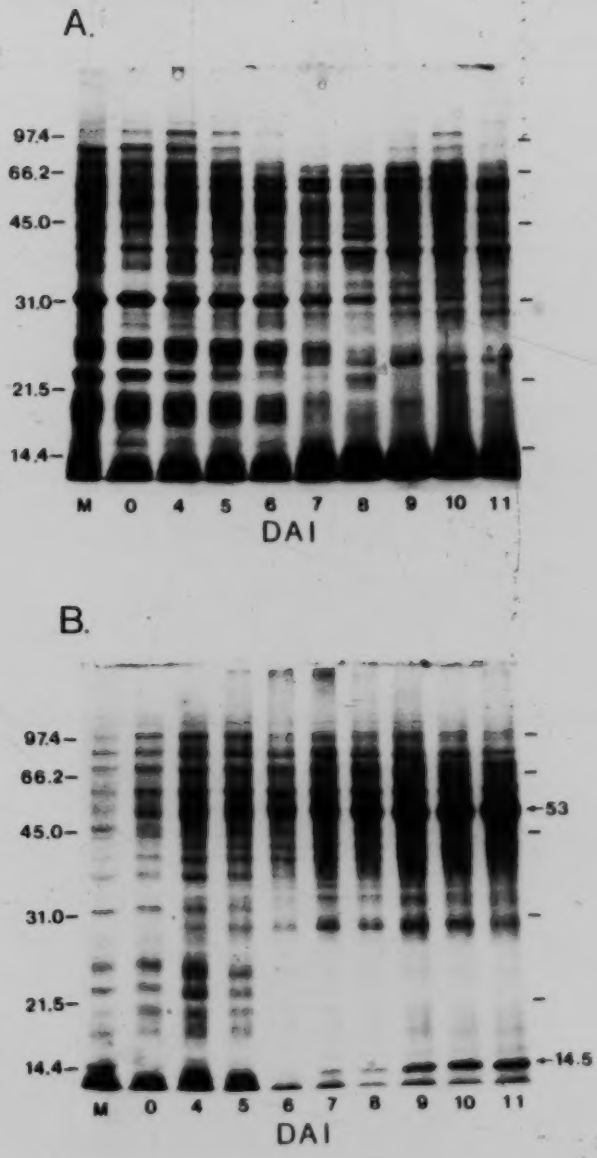
A, non-reduced; B, reduced; M, mature seed; 0, fully stratified seed; 4-11, days after imbibition. Molecular weights of protein standards in kDa indicated adjacent to gels. Arrows indicate significant proteins with their corresponding molecular masses.

Figure 8 **Coomassie stained SDS-PAGE profiles of insoluble protein fractions from the embryo following imbibition.**



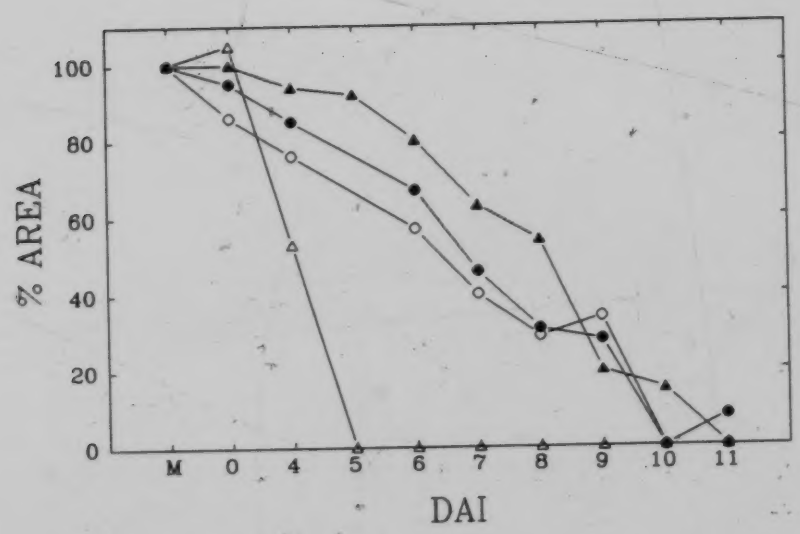
Labels as for Figure 7.

Figure 9 **Coomassie stained SDS-PAGE profiles of soluble protein fractions from the embryo and megagametophyte following imbibition.**



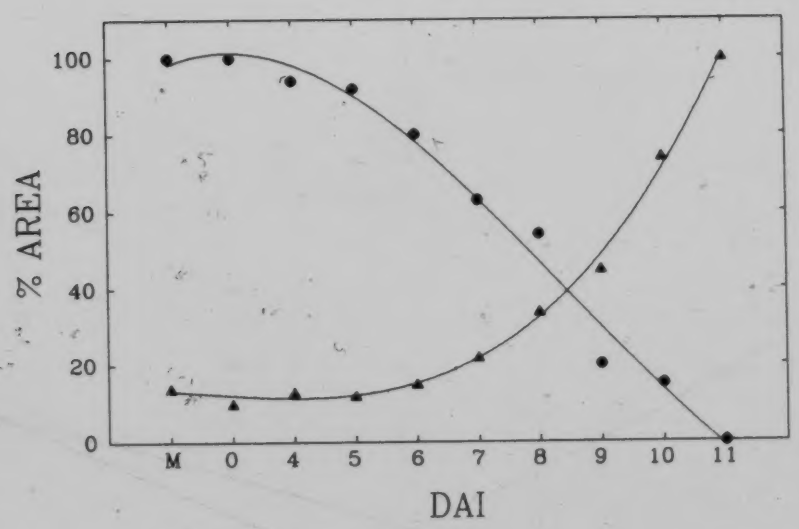
A, megagametophyte; B, embyro. Labels as for Figure 7.

Figure 10 Quantitative changes in specific seed storage proteins treated with ME following seed imbibition.



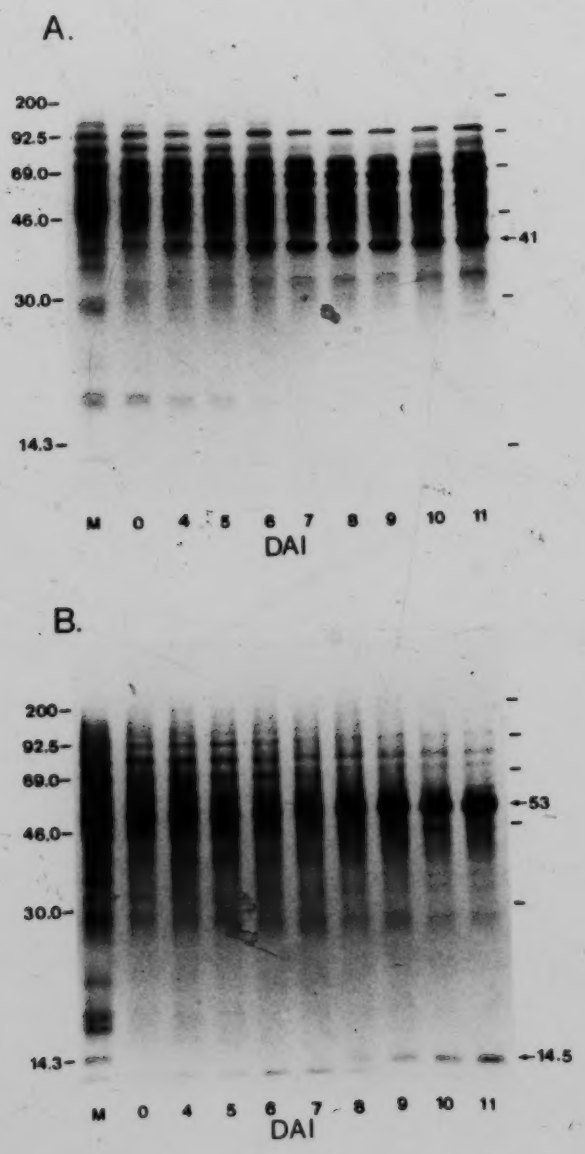
●, megagametophyte 47 kDa insoluble protein; ▲, megagametophyte 37.5 kDa insoluble protein; ○, embryo 47 kDa insoluble protein; △,embryo 37.5 kDa insoluble protein. Data were obtained from gels in Figure 7 and 8 by scanning densitometry. Labels as for Figure 2.

Figure 11 **Quantitative data obtained for the 37.5 kDa reduced and 37 kDa non-reduced megagametophyte insoluble proteins following seed imbibition.**



●, 37.5 kDa insoluble protein (+ME); ▲, 37 kDa insoluble protein (-ME). Data was obtained by scanning gels in Figure 7. Labels as for Figure 2.

Figure 12 Fluorographs of [^{35}S]-methionine labelled soluble proteins from the megagametophyte and embryo during imbibition subjected to one-dimensional electrophoresis.



A, megagametophyte; B, embryo; Labels as for Figure 7. Molecular weights of methylated protein standards are indicated adjacent to gels. Arrows indicate significant proteins with their corresponding molecular masses.

Figure 13A Fluorographs of [^{35}S]-methionine labelled soluble megagametophytic proteins from M (mature), 0 (fully stratified) and 1-4 DAI seeds subjected to two-dimensional electrophoresis.

Range of pH for first dimension indicated at top. Molecular weights in kDa of methylated standards run in the second dimension are adjacent to gels. ◯, proteins synthesized during germination; □, proteins synthesized after germination; ▯*, constitutively synthesized (except in the mature seed) proteins static with time; ▯', constitutive proteins decreasing with time; ▯+, constitutive proteins increasing with time; △, proteins synthesized only in the mature seed.

* note: ▯, indicates the absence of constitutively synthesized proteins static with time in the mature seed.

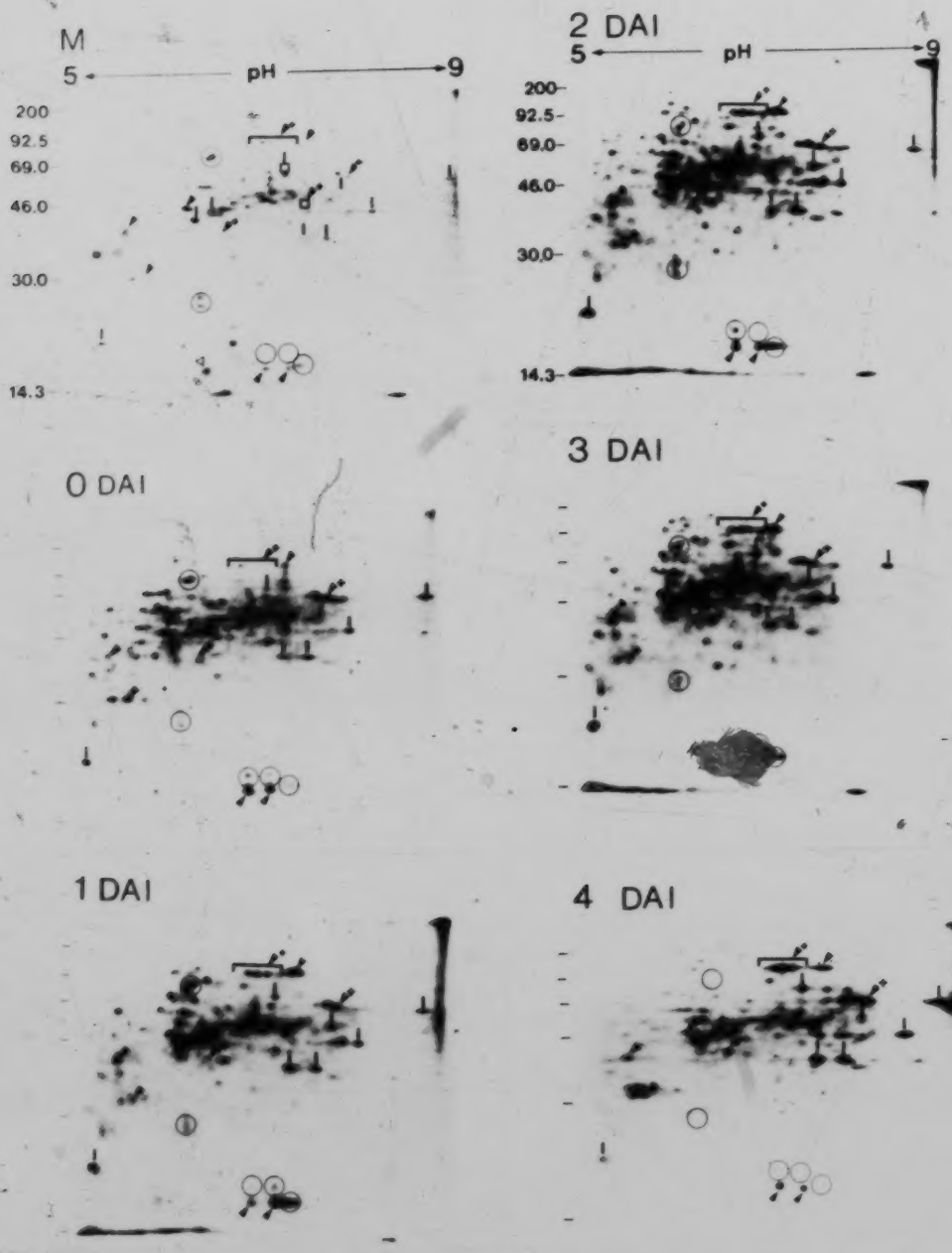

M
5
pH
9
200
92.5
69.0
46.0
30.0
14.3
2 DAI
5
pH
9
200
92.5
69.0
46.0
30.0
14.3
0 DAI
3 DAI
1 DAI
4 DAI

Figure 13B Fluorographs of [^{35}S]-methionine labelled soluble megagametophytic proteins from 5, 6, and 8 DAI seeds subjected to two-dimensional electrophoresis.

Labels as for Figure 13A.

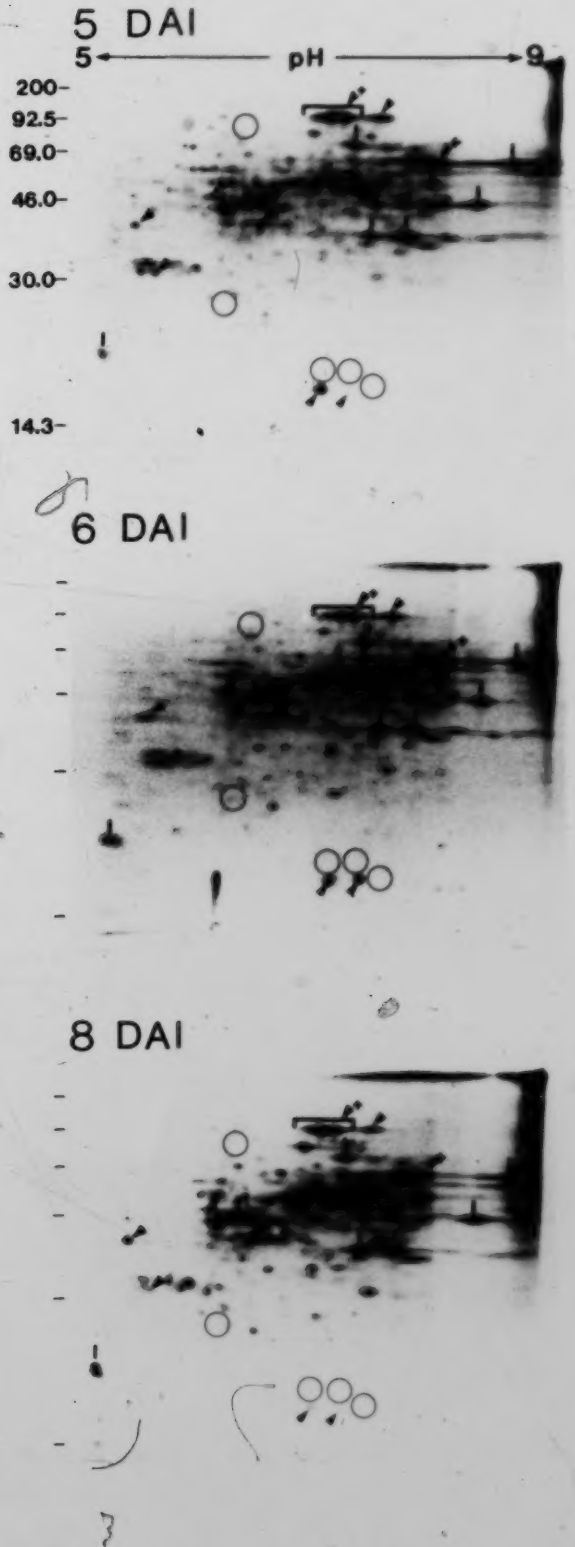

5 DAI
5
pH
9
200-
92.5-
69.0-
46.0-
30.0-
14.3-
6 DAI
8 DAI

Figure 14A Fluorographs of [^{35}S]-methionine labelled soluble embryonic proteins from M (mature), 0 (fully stratified) and 1-4 DAI seeds subjected to two -dimensional electrophoresis.

Labels as for Figure 13A.

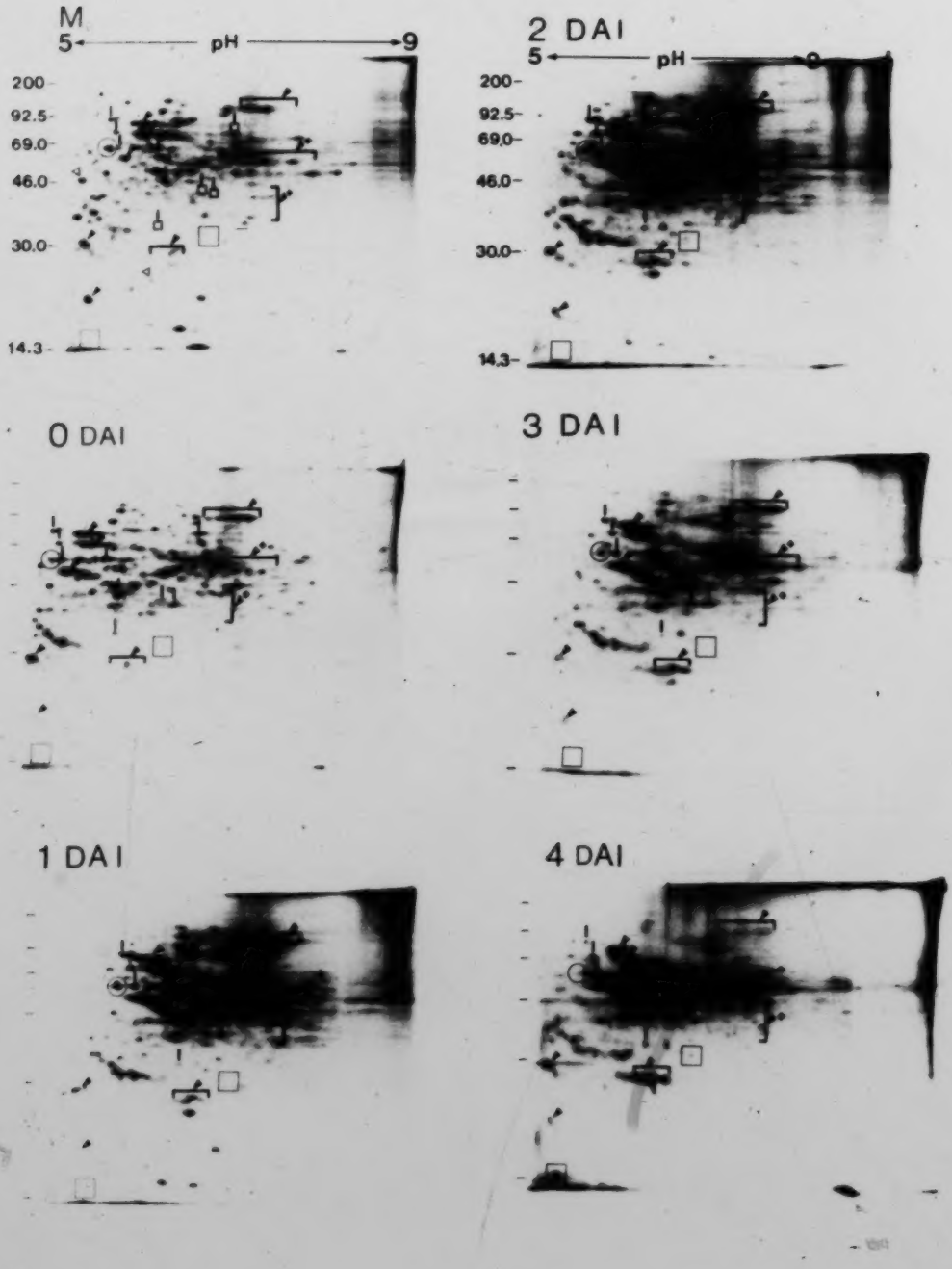
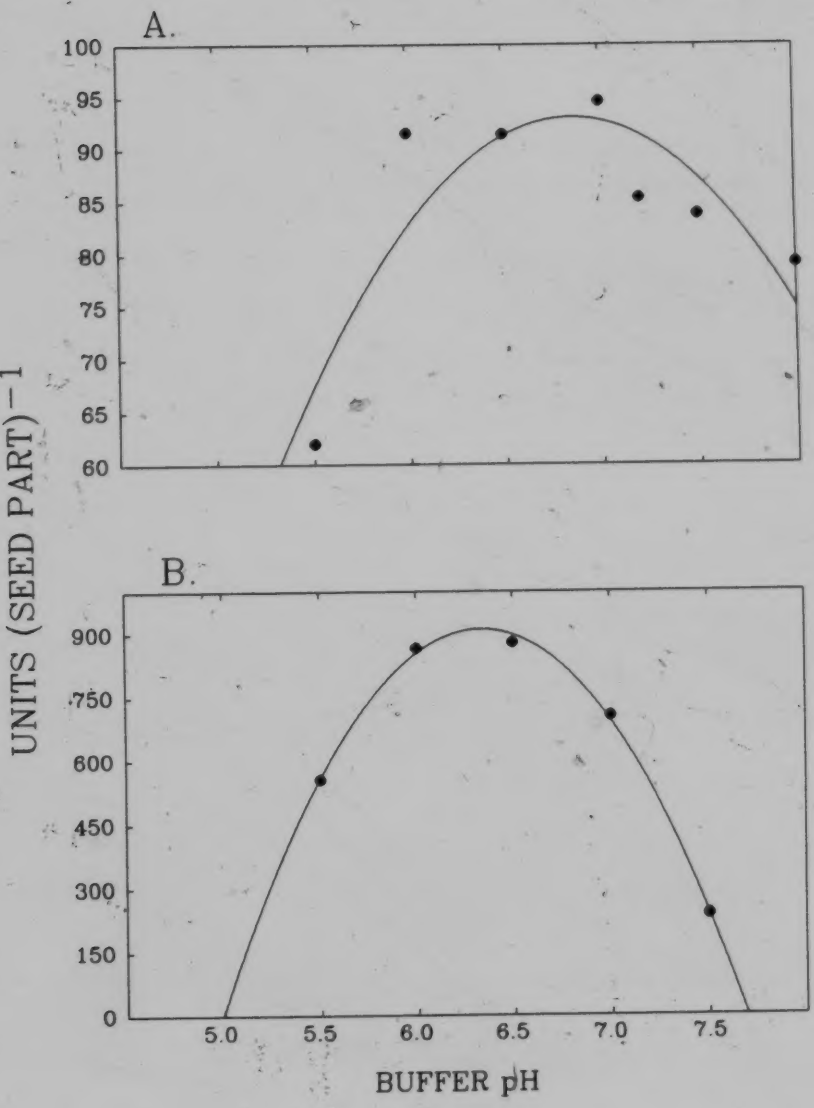

M
5
pH
9
200
92.5
69.0
46.0
30.0
14.3
2 DAI
0 DAI
3 DAI
1 DAI
4 DAI

Figure 14B Fluorographs of [^{35}S]-methionine labelled soluble embryonic proteins from 5, 6, and 8 DAI seeds subjected to two-dimensional electrophoresis.

Labels as for Figure 13A.

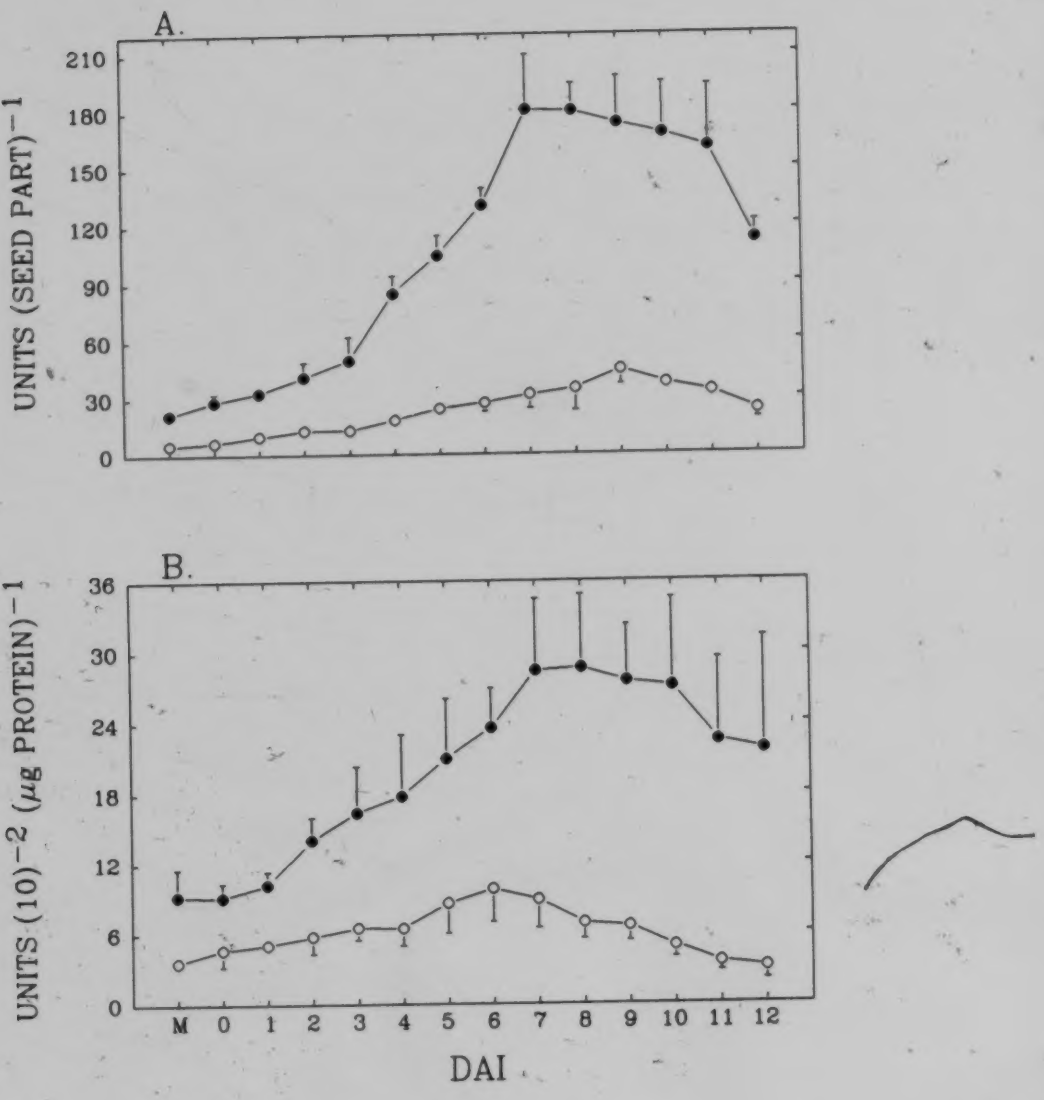
5 DAI
5
pH
9
200
92.5
69.0
46.0
30.0
14.3
6 DAI
8 DAI

Figure 15 The effect of pH on catalase and isocitrate lyase activity.



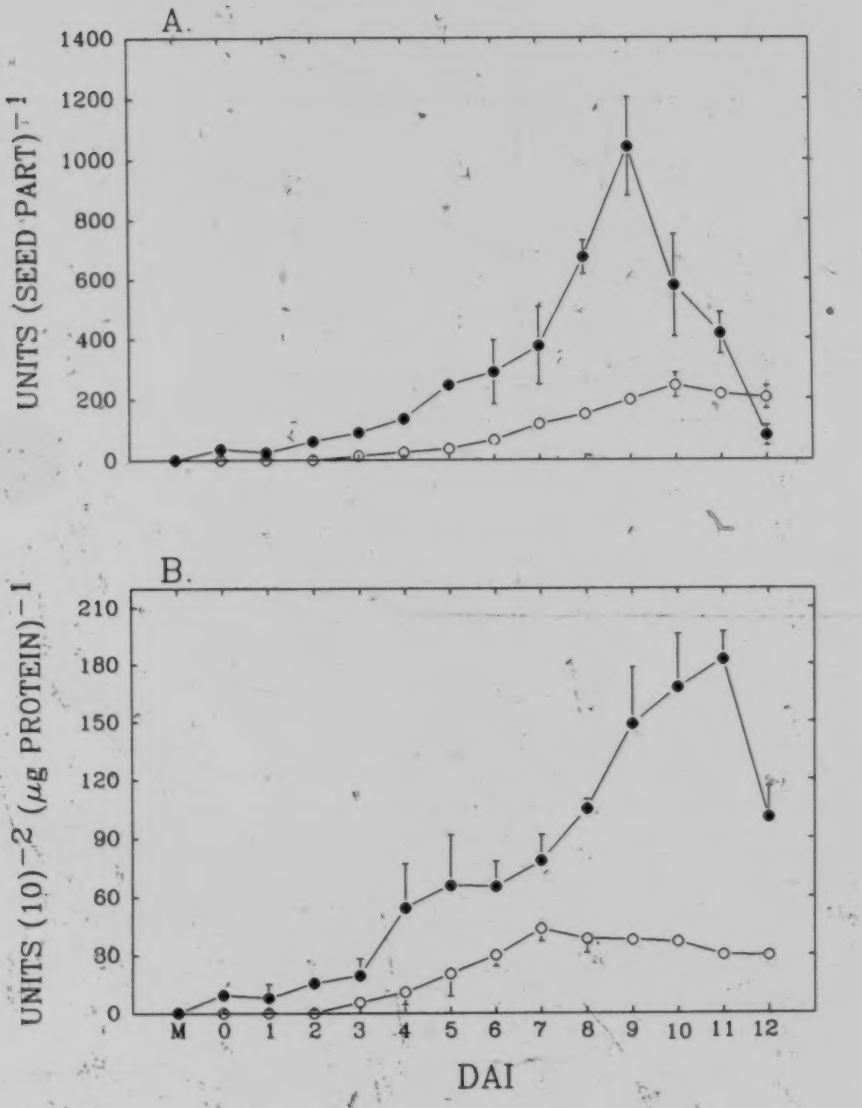
A, catalase; B isocitrate lyase. Optimums determined from whole germinated seeds.

Figure 16 Changes in catalase activity following imbibition in the megagametophyte and embryo.



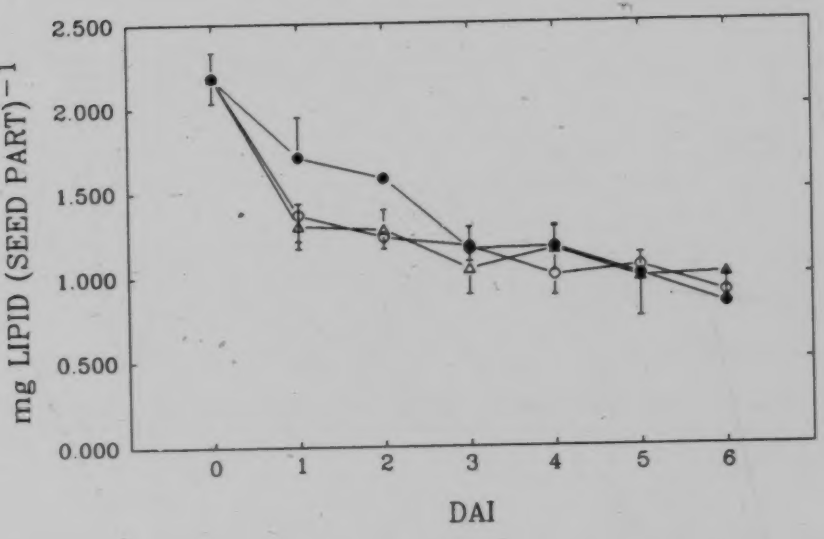
A, units per seed part; B, units per μg protein. Each data point is a mean of 3 determinations ± SD. Labels as for Figure 2.

Figure 17 Changes in isocitrate lyase activity following imbibition in the megagametophyte and embryo.



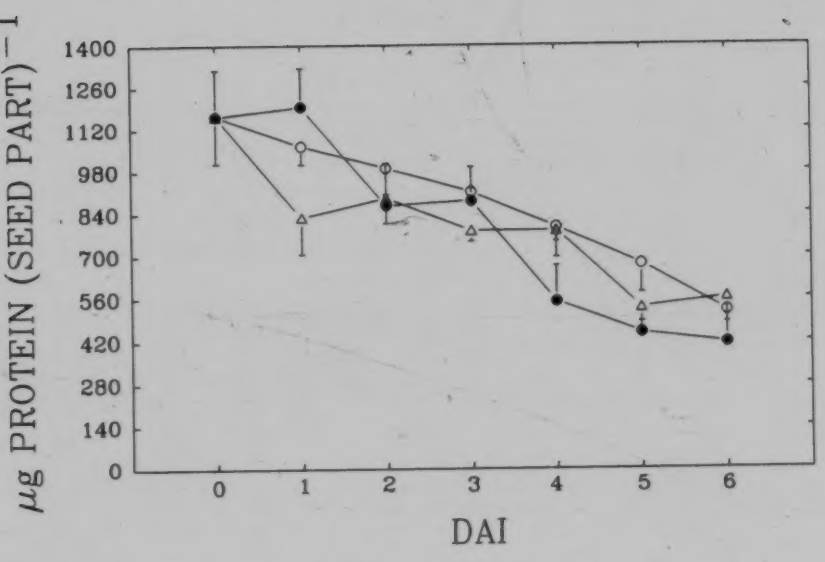
A, units per seed part; B, units per μg protein. Each data point is a mean of 3 determinations ± SD. Labels as for Figure 2.

Figure 18 **Effect of the embryo on the hydrolysis of lipid reserves in the megagametophyte during imbibition.**



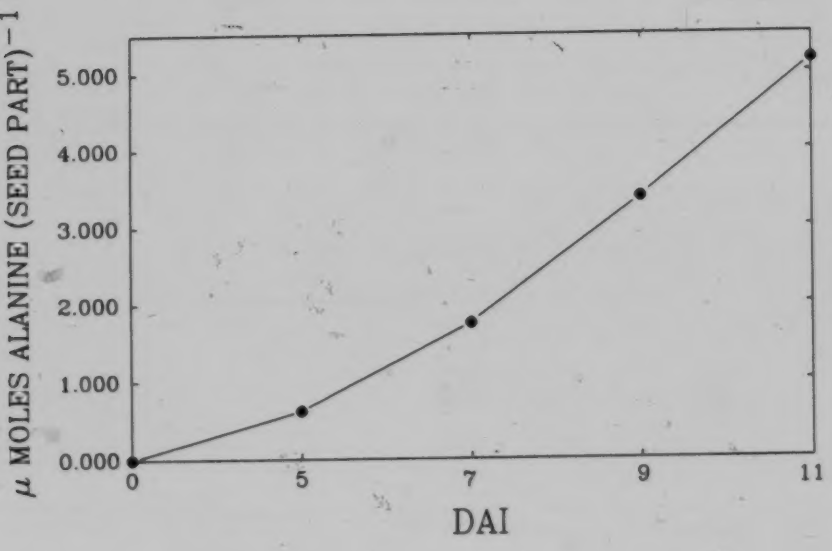
●, whole seed (minus seed coat); o, embryo removed, megagametophyte placed with surface usually proximal to the embryo down; △, embryo removed, megagametophyte placed with surface usually distal to the embryo down. Each data point is a mean of 2 determinations ± SD.

Figure 19 **Effect of the embryo on the hydrolysis of protein reserves in the megagametophyte during imbibition.**



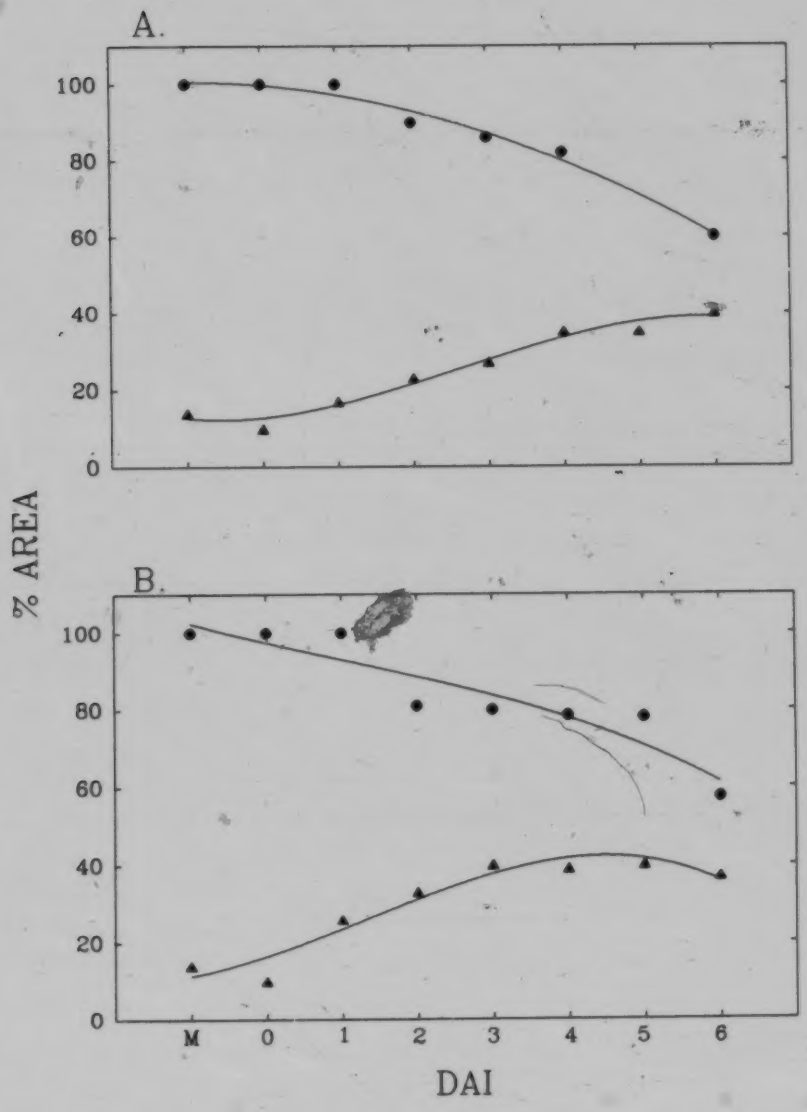
Symbols as for Figure 18. Each data point is a mean of 3 determinations ± SD.

Figure 20 Exportation of soluble amino acids into agar from megagametophyte halves following imbibition.



O, fully stratified seed. Values on X-axis are normalized to whole seed DAI radicle size classes to facilitate comparison with Figure 5. Each data point is a mean of 3 determinations.

Figure 21 **Effect of the embryo on quantitative data obtained for the 37.5 kDa megagametophytic insoluble protein and the 37 kDa product following seed imbibition.**



A, plus embryo; B, minus embryo, symbols as for Figure 11. Data was obtained by scanning Coomassie stained gels (not included).

Figure 22 The effect of the embryo on fluorographs of [^{35}S]-methionine labelled soluble megagametophytic proteins from seeds imbibed for 12 hours to 3 days subjected to two-dimensional electrophoresis.

Range of pH for first dimension indicated at top. Molecular weights in kDa of methylated standards run in the second dimension are adjacent to gels. ', proteins not affected by embryo removal; ', proteins synthesized only in the presence of the embryo, or only in the absence; ○, proteins synthesized to a lesser extent when embryo removed; □, proteins synthesized to a greater extent when embryo removed.

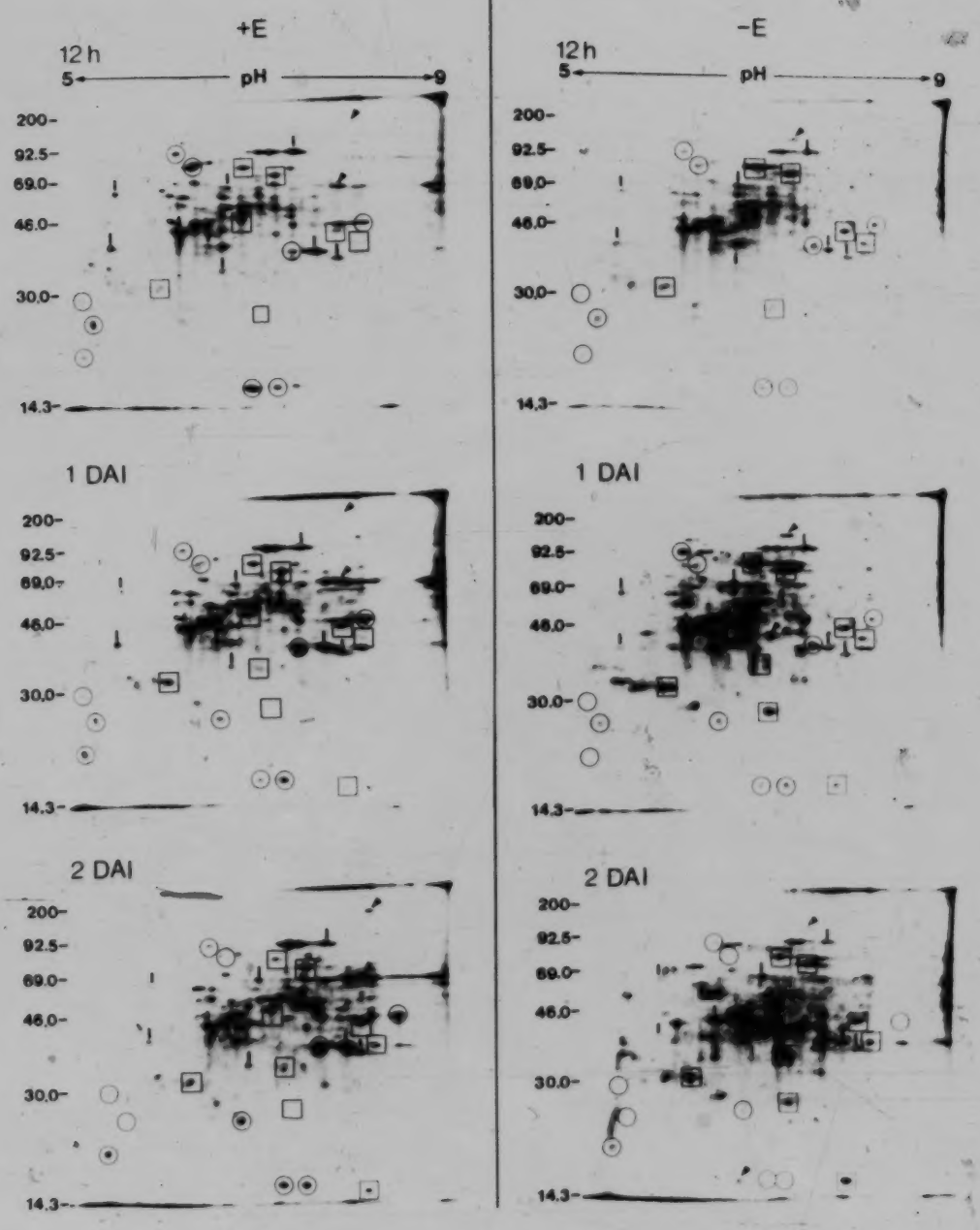
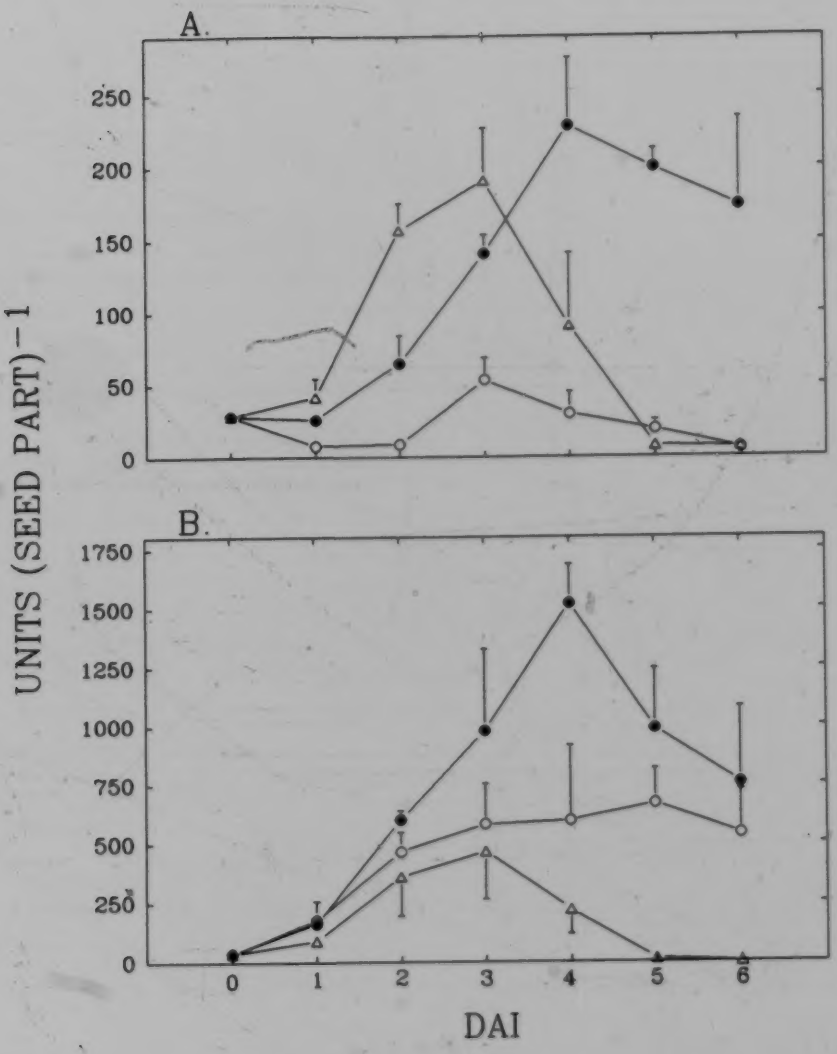
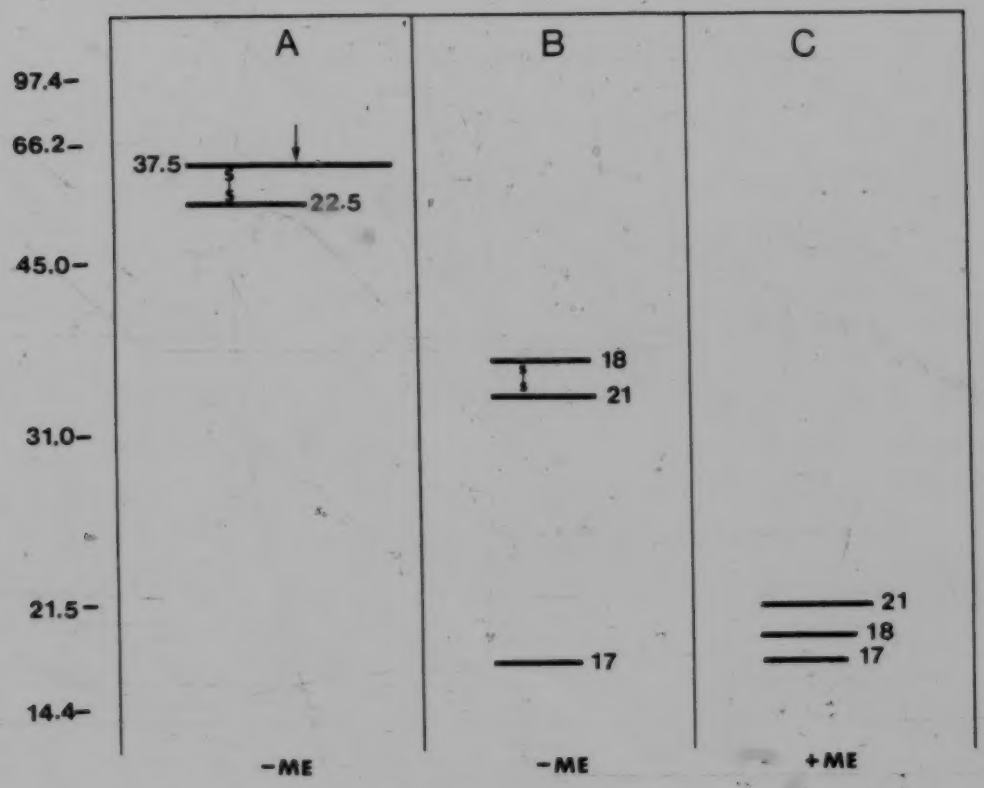

+E
12 h
5
pH
9
200-
92.5-
69.0-
46.0-
30.0-
14.3-
1 DAI
2 DAI
-E

Figure 23 **Effect of the embryo on catalase and isocitrate lyase activity in the megagametophyte during imbibition.**



A, catalase, B, isocitrate lyase. ●, whole seed (minus seed coat); o, embryo removed, megagametophyte placed with surface usually proximal to the embryo down; △,embryo removed, megagametophyte placed with surface usually distal to the embryo down. Each data point is a mean of 3 determinations ± SD.

Figure 24 Diagrammatic scheme of the hydrolysis of the 60 kDa subunit.



Temporal sequence of hydrolysis is from A to B to C. S-S, represents a disulphide bridge; ↓ represents point of enzymatic cleavage. Numbers at left margin represent molecular weight standards. Numbers beside bands represent molecular masses of proteins.

IV. DISCUSSION

Portions of this thesis dealing with the mobilization of protein and lipid reserves, changing protein patterns and protein synthesis have been accepted for publication in Physiologia Plantarum (Groome & Gifford 1991 in press).

The following discussion compares the results of characterizing the biochemical events occurring during germination and early seedling growth in loblolly pine seed to that found in other plants, and where possible, to other conifers. Patterns of development in protein synthesis and enzyme activity during the study period are also discussed. Mechanisms proposed for similar changes found in other species are presented, and their relevance to this research discussed. Finally, suggestions for further research are given.

4.1 FACTORS CONTROLLING GERMINATION

It is apparent from the results in Figure 1 and Table 3, that physical and physiological factors influence the germinability of loblolly pine seeds. An increase in germination corresponding to periods of cold moist stratification has been shown in many conifers. For example, *P. monticola* requires a stratification period of 90 days for consistent and high levels of germination to occur. Hoff (1987) suggested that a combination of inhibitors and promoters are involved in this process. Inhibitors of proteinases have been found in resting seed of *P. silvestris*

which disappear during stratification, allowing protein hydrolysis to occur (Salmia 1981). This disappearance may be a result of leaching out or enzymatic breakdown during stratification (Halmer & Bewley 1979). Germination promoters may also be synthesized or transported during this period (Nikolaeva 1968). It has been reported that growth regulators such as gibberellic acid and kinetin promote germination of stratified loblolly pine seed (Biswas *et al.* 1972). Paul *et al.* (1973) found that unstratified loblolly seed contained no such growth substances, but had a high concentration of an unknown inhibitor. Following stratification, the inhibitor was undetected while high levels of gibberellin and auxin-like substances were present.

The significance of seed coat and nucellus removal on percent germination of pine seeds can be found in the literature. Stone (1957), Kozlowski & Gentile (1959) and Barnett (1976) all concluded that the seed coat forms a barrier to the entry of oxygen into the seed. Baron (1978) believed that the thin papery layer of the seed coat was also important in restricting water uptake. Both oxygen and water are necessary for metabolic processes to occur, therefore removal of the whole seed coat should hasten germination, which was the case for loblolly pine in this study. Removal of the nucellus is probably also involved in accelerating germination. The meristematic tissue of the

radicle is exposed when the nucellus is removed, allowing more efficient water uptake (Hoff 1987).

4.2 SEED RESERVES

Loblolly pine appears to have seed reserves, distinguished by their rapid depletion as the radicle emerges from the seed, which are characteristic in type and abundance of those of many other gymnosperms. Similarities in the temporal depletion of reserves are also apparent. Although the length of the germinative period and the rate of seedling growth varies notably between species, consistencies can be seen in relation to radicle emergence.

4.2.1 Lipids

Lipids are the most abundant food reserve in loblolly pine embryos and megagametophytes, as is the case in *Abies alba* (Kovac & Vardjan 1981) and *Pseudotsuga menziesii* (Ching 1966). In all three species, the bulk of the lipid reserve is situated in the megagametophyte. It appears that lipid depletion in this tissue commences during stratification in *P. menziesii* and *P. taeda*, and continues throughout germination and early seedling growth. This is distinct from the pattern seen in *Picea*, where lipid utilization is postponed until the radicle has emerged (Cyr *et al.* 1991 in press). It seems characteristic of conifer embryos for the accumulation of lipids resulting from growth to be preceded by a drop in total lipid, likely due to the initial decomposition of embryonic reserves.

In *R. communis* (Beevers 1969) and *Pinus edulis* (Murphy *et al.* 1991) lipid reserves from the nutritive tissue are transported to the embryonic axis in the form of sucrose. The sucrose is then thought to be converted to starch in the embryo of *Pinus edulis* (Murphy *et al.* 1991). It would be of interest to pursue the fate of lipid reserves in loblolly pine, and assess the relationship between reserve hydrolysis and product accumulation.

4.2.2 Proteins

Although the bulk of storage reserves are lipids, proteins also play a large part in supplying nutrition to loblolly pine embryos. Total protein constitutes 15% of the fresh weight of the megagametophyte and 17% of the embryo, consistent with the range observed for other gymnosperm species (Gifford 1988, Kovac & Kregar 1989). Analogous to other species, the megagametophyte houses nearly all the seed protein in loblolly pine.

The major storage proteins in many conifers (Gifford 1988, Lammer & Gifford 1989, Misra & Green 1990, Flinn *et al.* 1991, Green *et al.* 1991) are characterized by their solubility in buffer solutions containing SDS or urea. In loblolly pine, 75% of the proteins in the megagametophyte belong to this category, comparable to the proportion of insoluble proteins in many pines (58-80% of the total protein, Gifford 1988). Approximately 85% of buffer-insoluble proteins in loblolly pine are similar to the crystalloid proteins found

in other species. Crystalloid proteins have a hexameric quaternary structure. Each subunit is a heterodimer, and the two polypeptides are linked by disulphide bridges (Gifford 1988, Gifford & Tolley 1989, Jensen & Berthold 1989, Lammer & Gifford 1989, Misra & Green 1990). The characteristic disulphide bridge linking subunit polypeptides is evident in the 60 kDa protein in both tissues, which is replaced by 37.5 and 22.5 kDa bands upon treatment with ME. These molecular masses correspond closely to heterodimers and their subunit polypeptides in other species of pine (Gifford 1988). In addition, a 47 kDa insoluble protein, comprising 8% of the insoluble proteins of mature loblolly seed, does not appear to have subunit polypeptides linked by disulphide bridges and is not affected by ME.

Soluble proteins contribute to seed storage reserves in varying degrees in different conifer taxa. Low molecular mass soluble proteins which appear to have a storage function have been detected in various pine species (Gifford 1988, Lammer & Gifford 1989). These proteins generally have a molecular mass between 24 and 32.5 kDa. Mature loblolly pine seeds also contain a low molecular mass soluble protein (32 kDa) which disappears rapidly upon imbibition, indicative of a storage function. In at least four studies to date on conifers (*Pinus albicaulis*, *P. glauca* and species of *Abies*), soluble proteins comprise a more significant portion of the reserve than the insoluble fraction (Gifford 1988,

Gifford & Tolley 1989, Kovac & Kregar 1989, Jensen & Lixue 1991).

The conversion to soluble amino acids is critical for the transportation of the energy stored in protein reserves to the embryo. Normally reserves in the protein body are in an insoluble form and can not be transported as such, making their hydrolysis into soluble amino acids essential. This has been determined to be a multi-faceted process. Sulfhydryl endopeptidases often play the initial role in hydrolysis of storage reserves (Chrispeels & Boulter 1975, Baumgartner & Chrispeels 1977), with other enzymes acting cooperatively to produce smaller peptides and amino acids (Salmia 1981).

A hypothetical sequence for the hydrolysis of the crystalloid subunits in loblolly pine can be deduced by following the insoluble profiles during germination and early seedling growth (Figure 7). Coincident with the disappearance of the 60 kDa subunit is the appearance of proteins with molecular masses of 37 and 17 kDa. The additive molecular masses of these two proteins is close to 60. In addition, neither protein incorporated [^{35}S]-methionine during pulse-labelling (gels not shown). It is possible that these two proteins are the initial hydrolysis products of the 60 kDa subunit. Reduction of these hydrolysis products with ME results in the disappearance of only the 37 kDa protein (Figure 7B), and the appearance of two proteins with molecular masses of 21 and 18 kDa. The 17

kDa protein does not appear to be affected by ME treatment. The first enzyme to be involved in the hydrolysis of the crystalloid subunit could cleave the 37.5 kDa subunit polypeptide, producing a 17 kDa peptide and a 37 kDa heterodimer. This heterodimer is comprised of two proteins with molecular masses of 21 and 18 kDa. This hypothesis is represented diagrammatically in Figure 24. It appears that subsequent action of peptidases results in very small peptides or amino acids since no other unlabelled soluble or insoluble products are detected.

The conspicuous lack of products of reserve hydrolysis in electrophoretic patterns of loblolly pine embryo proteins, suggests that these storage proteins are very different than their counterparts in the megagametophyte. Another putative explanation is that hydrolysis occurs so rapidly in the embryo that accumulation of breakdown products is at the expense of new protein synthesis. In *P. contorta*, insoluble protein reserves in the embryo are broken down very quickly (Lammer & Gifford 1989). Rapid hydrolysis of embryonic crystalloid proteins has also been documented for *R. communis* (Kermode *et al.* 1985).

4.2.3 Embryo dependance on the megagametophyte for reserves

Evidence that the products of hydrolysis in the megagametophyte are indeed transported to the embryo in loblolly pine is apparent from the data on amino acid accumulation in the embryo and megagametophyte (Figure 5).

A rise in the megagametophytic amino acid pool does not coincide with protein hydrolysis in this tissue, similar to results obtained for *P. contorta* (Lammer & Gifford 1989). The embryo on the other hand shows a very large increase in amino acids during this period. This is not caused solely by the breakdown of storage proteins in the embryo; as shown in Figure 5, embryos grown *in vitro* accumulate only a very small amount of amino acids. Amino acids apparently move from an area of high concentration in the megagametophyte (source), to one of low concentration in the embryo (sink). Utilization of the amino acids by the embryo maintains the concentration gradient and the source to sink flow.

The embryo mobilizes its own reserves initially as shown by the rapid insoluble protein depletion (Figure 10) occurring before germination, then depends on amino acids transported from the megagametophyte. It is interesting that the seedling, after only 1 day following radicle emergence, has synthesized chlorophyll. Following this greening of tissue in the embryo, lipids are still hydrolyzed in the megagametophyte, indicating a possible delayed nutritive role for the megagametophyte. In *A. araucana*, Cardemil & Reinero (1982) concluded that the megagametophyte is not the main source of nutrition for the embryo for the first 4 days after imbibition. The embryo can develop into a seedling relying exclusively on its own reserves; the megagametophyte may play a role later in development. In other conifers, reserves in the embryo

begin the hydrolytic process before any detection of similar patterns in the megagametophyte (Simola 1974, Kovac & Kregar 1989). Loblolly pine seed embryos demonstrate this independence during early imbibition; isolated embryos show normal growth on water agar for a few days in the absence of the megagametophyte (Gifford unpublished data).

4.3 EVIDENCE FOR THE REGULATION OF RESERVE MOBILIZATION

Criteria such as amino acid incorporation into protein or the development of enzyme activity can be used to establish that reserve mobilization is regulated by protein synthesis and/or enzyme activation.

4.3.1 Changes in protein synthesis

It is clear that mature loblolly pine seeds have the ability to synthesize proteins in the first 3 hours of imbibition (lane M Figure 12). This early synthesis is likely programmed by preformed mRNA. One must be careful not to exclude the possibility that the synthesis of many proteins within that 3 hours is directed by newly formed mRNA however. The synthesis of eukaryotic mRNA takes only 15 to 20 minutes (Aspart *et al.* 1984) and in wheat, 90% of the mRNA is replaced in 2 hours (Smith *et al.* 1974). Reducing the labelling period prior to electrophoresis to 15 minutes would delineate those proteins translated from preformed mRNA. Subsequent changes in protein synthesis in loblolly pine were likely due to *de novo* mRNA synthesis.

By the completion of the stratification period, shifts in protein synthesis were especially pronounced in the embryo as seen by one-dimensional electrophoresis. The synthesis of four low molecular mass bands in the mature embryo was restricted following stratification. Further evidence that temporal regulation is precisely controlled was the relative increase in a 53 kDa protein initiated at the time of greening. This protein corresponds in mass to the large subunit of the essential photosynthetic protein ribulose bisphosphate carboxylase which would be needed in abundance once the radicle emerged from the seed coat.

Two-dimensional electrophoresis revealed that proteins synthesized during the duration of the experiment can be grouped into subsets defined by temporal appearance. Similar to subsets distinguished by Dure *et al.* (1983), the groups of proteins in loblolly pine seem to be correlated with major developmental changes occurring in the seed. Most can be included in a subset of proteins synthesized constitutively following stratification, which probably performs a maintenance function in the seed. Of significance is the absence of many of these proteins from the mature seed, indicating again the importance of stratification in the regulation of protein synthesis. The majority of proteins in loblolly pine in this first category have their relative amount of synthesis regulated. Other proteins belong to subsets that are synthesized only before or only after radicle emergence. Proteins synthesized only

following germination are predictably involved in seedling growth, while proteins only involved in germination would be confined to that period.

These three subsets of proteins were also seen by one-dimensional electrophoretic analysis of buffer-soluble proteins in *P. contorta* (Gifford *et al.* 1990). The transition between synthesis of protein subsets is triggered by the completion of germination in both species. Germination is a critical period of transition as seen by changes in protein synthesis.

Proteins involved in reserve hydrolysis should also show tight temporal regulation. Out of necessity, their synthesis would have to begin during germination to facilitate the rapid degradation of lipid and protein which is seen in so many species following radicle emergence (Dure *et al.* 1983). Antibodies for hydrolytic enzymes would be beneficial in elucidating exactly when synthesis commences, and if, or when, during early seedling growth synthesis is terminated.

4.3.2 Changes in enzyme activities

The continuous chemical assay used to detect ICL activity in this thesis is the preferred one (Vanni *et al.* 1990), as long as the pH used is not higher than 7.0. A more basic buffer is known to reduce the rate of formation of glyoxylate-phenylhydrazone, and may have affected the results of some previous studies (Murray & Adams 1980).

One oversight on my part was the effect of HPO_4^{-2} on ICL activity (Vanni *et al.* 1990). Phosphate buffers are generally no longer used in ICL assays because HPO_4^{-2} can inhibit activity. Due to the wide variety of techniques used to detect ICL activity, comparisons of actual units are virtually impossible, but general trends during the study periods can be compared.

While catalase and other glyoxylate cycle enzymes are detected in mature seed extensively throughout seed plants the plant kingdom, ICL activity is generally absent (Yamamoto & Beevers 1960, Lee *et al.* 1964, Smith *et al.* 1971, Doig *et al.* 1975, Miernyk *et al.* 1979, Bortman *et al.* 1981). The same holds true for loblolly pine seed. Various theories have emerged to explain this phenomenon in angiosperms. The seed may contain ICL in its tissues in an inactive form until substrate is present or inhibitors are removed. For example, isocitrate lyase is present but inhibited from its catalytic function in crude extracts from mature seed of sunflower (*Helianthus annuus*) (Theimer 1976, Fusseder & Theimer 1984, Allen *et al.* 1988), species of *Musa* (Surendranathan & Nair 1978) and *C. sativus* (Frevert *et al.* 1980). Upon purification of these extracts, which should remove any inhibitors present, ICL activity is detected.

In species of *Gossypium*, a lack of ICL activity is not attributable to inhibitors (Ihle & Dure 1972, Choinski & Trelease 1978, Miernyk *et al.* 1979); ICL is simply absent from the seed. The absence of the enzyme in the dry seed

suggests that the enzyme is synthesized *de novo* upon imbibition. Work done by Longo (1968) showed that in peanut (*Arachis hypogeae*), ICL is synthesized in the cotyledons after imbibition. *De novo* synthesis could be programmed by mRNA formed during seed maturation and activated upon rehydration (Ihle & Dure 1972, Khan *et al.* 1976, Tester 1976, Allen *et al.* 1988), or may be dependant on the synthesis of new mRNA (Hock & Beevers 1966, Smith *et al.* 1974, Radin & Trelease 1976, Weir *et al.* 1980). Whatever the cause for the lack of ICL activity, it could possibly play a regulatory role in lipid hydrolysis in loblolly pine. Although the other enzymes involved in hydrolysis may be present and active, all the enzymes must co-exist in their functional form before mobilization can be accomplished. ICL may therefore be a key regulatory enzyme.

In general, the kinetics of ICL and CAT in loblolly pine are very similar to those of other species. As found in all plants, ICL activity rises in the nutritive tissue as lipids are metabolized, followed by a drop as reserves are depleted. In loblolly pine there is a coordinate rise in catalase activity with the period of maximum reserve degradation, but activity does not fall off as abruptly in the megagametophytes. Because catalase is not restricted in its function to the glyoxysome and lipid hydrolysis, prolonged activity is expected for this enzyme.

It was interesting to find a relatively high level of ICL activity in the embryo of loblolly pine as germination

proceeded. This is contrary to what seen in *P. ponderosa* (Ching 1970) and *A. alba* (Kovac & Wrischer 1984); ICL was undetected in the embryo at all stages studied for both of these conifers. Kovac & Wrischer suggested that lipids were metabolized in a different fashion in the embryo; a functional glyoxylate cycle was not necessary. In contrast, the majority of monocots and dicots studied have detectable ICL activity in the seedling (Carpenter & Beevers 1959, Schrauwen & Sondheimer 1975, Davies & Pinfield 1980). The presence of ICL in the embryo of loblolly pine suggests that the embryo during germination is actively involved in metabolizing its own lipid reserves via the glyoxylate cycle, as well as receiving breakdown products from the megagametophyte.

4.4 THE ROLE OF THE EMBRYO

The assumption that the embryonic axis regulates processes in the megagametophyte is usually based on a reduction in reserve mobilization and hydrolytic enzyme activity upon axis removal. As mentioned previously, these observations result from either a deprivation of a necessary substance from the embryo, or feed-back inhibition in the megagametophyte. Obviously the removal of the embryo before imbibition does not affect the hydrolysis of reserves in the megagametophyte of loblolly pine. Megagametophyte lipid hydrolysis appears to be independent of any signal from the embryo, unless of course that signal is relayed earlier

during stratification. It would be interesting to study whether the latter situation is true by removing the embryo at different times during stratification and examining the effect on reserve mobilization.

Of interest was the effect that embryo excision had on enzyme activities in the megagametophyte. For both ICL and CAT, removal of the axis, while replacing the role of the embryo as a sink with agar, resulted in lowered optimum activity. This suggests that an embryonic signal is needed for optimum activity of these two enzymes in the megagametophyte. The amino acid data in Figure 20 casts a shadow on the effectiveness of replacing the embryo with agar as a sink however. The amount of amino acids exported from the megagametophyte halves into the agar should be equal to, if not greater than, the amino acids accumulated in the embryo *in vivo* (Figure 5). The apparent amount of reserves translocated out of the megagametophyte was considerably lower when agar was used as a sink, yet the rate of protein hydrolysis was unaffected. In the case of lipid mobilization, Ching (1970) suggested that the continuous removal of sugars by the embryo keeps the glyoxylate cycle operational in the megagametophyte. Murray (1991 in press) has demonstrated that the sucrose transported to the embryo from the megagametophyte is subsequently converted to starch. This would prevent sucrose from building up and maintain a large concentration gradient between the megagametophyte and the embryo.

Theoretically agar would be inefficient as a sink because it can not actively convert the breakdown products like the embryo can. Perhaps an inactive sink like the agar used in these experiments allows enough accumulation of amino acids and sucrose to reduce hydrolytic enzyme activity, but not enough to affect total mobilization of reserves. A more plausible explanation is that enzyme assays of cell-free extracts do not indicate accurately the processes occurring in the intact seed.

At first glance, one would rule out feed-back inhibition as a mechanism of controlling the rate of lipid and protein hydrolysis in loblolly pine; removing the embryo as a sink did not change the rate of hydrolysis. To decide whether or not the sink was effectually removed is difficult however. Pools of exudate did accumulate on the agar around the megagametophyte halves even when the surface usually adjacent to the embryo was placed upwards. Perhaps the agar was still partially acting as a sink for the end-products and mobilization was therefore not inhibited. If the sink was only partially removed by the method used in this thesis, the accumulation of products could again have been sufficient to cause a reduction in enzyme activities. Again, a more likely explanation for this incongruency is the inefficiency of assaying cell-free extracts.

Studies on the role of the embryo in controlling metabolic processes in the nutritive tissue of seeds involves complicated interactions which are difficult to

isolate under laboratory conditions. The extensive differences seen in various species may result strictly from the use of different techniques. Murray & Adams (1980), for example, showed that even sterilization procedures can affect enzymatic results. The introduction of wounds when excising the embryo may introduce artifacts, and the dependability of cell-free extracts to reflect processes occurring in the whole seed may also be questioned.

Although the effect of embryo removal on enzyme activity remains inconclusive in loblolly pine, there is no doubt that removal of the embryo affects protein synthesis. A shift in pattern occurs almost immediately. Whether these shifts are also artifacts of technique, or reflect changes in the synthesis of hydrolytic enzymes remains to be clarified.

4.5 FUTURE RESEARCH

The many questions left unanswered about the regulation of reserve hydrolysis in this system demonstrates the need for antibodies and cDNA probes for specific proteins and mRNA. Antibodies would allow precise quantification of specific proteins, eliminating the need to assay cell-free extracts which can often be misleading and inaccurate. The presence or absence of enzymes such as ICL in the mature seed of loblolly pine could be clarified, and the timing of synthesis investigated. If specific proteins were found to be synthesized *de novo* upon imbibition, cDNA probes in

conjunction with *in vitro* translation could be used to assess whether synthesis is directed by preformed or newly synthesized mRNA. Determining the role of the embryo in regulating reserve mobilization in the megagametophyte would also be facilitated by the availability of antibodies and probes.

With this research as a solid foundation, studies may now focus on quantifying temporal changes in the mRNAs corresponding to enzymes involved in hydrolysis. The results from this study in conjunction with data accumulated on protein levels using antibodies will give us greater insight into the level at which gene expression is controlled. Because of the similarities in patterns of development among conifer species, results on the control of reserve mobilization in the loblolly pine system will significantly increase our understanding of conifer germination and early seedling growth in general.

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